

Microarray standards at last

Not a moment too soon, the microarray community has issued guidelines that will make their data much more useful and accessible. *Nature* and the Nature research journals will respond accordingly.

You read a paper with a fascinating conclusion about the expression of several genes. You decide to use some of the same experiments on your system of choice. But when you wade through hundreds of pages of supplementary information, you find that crucial details needed for replication are missing.

Welcome to the exciting but frustrating world of DNA microarray research. Microarrays are plastic or glass chips spotted with tiny amounts of thousands of probes, used to query the activity levels of that many genes in any tissue or organism at one time. Variables in every step of the experiment often make cross-paper comparison virtually impossible. Microarray papers also pose a considerable strain on the refereeing process; the vast amounts of data mean that critical review is a monumental task.

Yet referees sometimes feel they are not given enough details, leading cautious reviewers to think that they must reanalyse the primary data set. In other cases, the primary data provided are in proprietary software and so are impossible to comment on. Many journals allowed authors to put the huge data files on their own websites for the review process, until it became clear that unscrupulous authors compromised the anonymity of referees by tracking who had visited the website.

In a move to remedy these problems, the international Microarray Gene Expression Data (MGED) group has written an open letter to scientific journals proposing standards for publication. Other members of the microarray community welcomed these steps, designed to clarify the Minimal Information About a Microarray Experiment (MIAME) guidelines (*Nature Genetics* **29**, 365–371; 2001).

For authors, the proposal provides a checklist of variables that should be included in every microarray publication, at http://www.mged.org/Workgroups/MIAME/miame_checklist.html. This checklist, with all variables completed, would be supplied as supplementary information at the time of submission. The MGED group suggests that journals require submission of microarray data to either of two databases emerging as the main public repositories: GEO (www.ncbi.nlm.nih.gov/geo/) or ArrayExpress (www.ebi.ac.uk/arrayexpress).

Harried editors can rejoice that, at last, the community is taming the unruly beast that is microarray information. Therefore, all submissions to *Nature* and the Nature family of journals received on or after 1 December containing new microarray experiments must include the mailing of five compact disks to the editor. These disks should include necessary information compliant with the MIAME standard. The information must be supplied in a format that could be read by widely available software packages. Data integral to the paper's conclusions should be submitted to the ArrayExpress or GEO databases, with accession numbers where available, supplied at or before acceptance for publication.

How much data should authors provide to the community? Specifically, do other researchers really need to recreate the exact microarray just to test the expression level of a few key genes, which could presumably be done through other methods? Perhaps with further evolution and standardization of microarray technology, the need to specify so many variables will decrease, but the MGED standards are surely appropriate for the current state of the field. ■

Dolly, Bikini and syncopated angst

A new piece of music theatre is an unusually direct appraisal of science's outcomes by two outstanding artists.

Imagine four hands clapping in unison a fast syncopated rhythm that lasts just six quick beats. Repeat 12 times. Have one pair of hands shift the pattern in phase by half a beat, keeping the rhythm unaltered. The result to the listener is an emergent clapping pattern that is still fast and syncopated but quite different. Repeat 12 times, shift again, repeat, shift and so on until the original pattern re-emerges at the end of the cycle.

Clapping Music was written by Steve Reich in 1972, and represents the most distilled presentation of a style that he has embodied in many instrumental combinations. Requiring enormous concentration and skill from musicians, Reich typically deploys blocks of musical patterns of rhythm and harmony that unfold like a machine but are unpredictable and artistically judged, and can compel and delight any ear that has adapted to them. A true original, Reich has proved both popular and influential for the past 20 years.

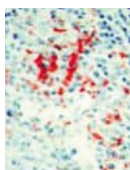
Three Tales is Reich's latest excursion in this style. Completed in May, it is now on international tour (see www.steverreich.com) and will be produced as a DVD. But it is more than pure music that filled a large theatre in London for four nights last week. The work is a highly integrated combination of music with a video with soundtrack. It addresses explicitly the significance for humanity of technology—in

particular, of the Hindenberg airship disaster, the Bikini Atoll test of the fission bomb, and Dolly the cloned sheep.

The video, by the pioneering video artist Beryl Korot (Reich's wife) takes images, newspaper cuttings and interviews of people involved in the events, and quotations from the Book of Genesis about responsibilities and power bestowed on mankind at the moment of creation. In the first section, the music ranges from keening harmonies in response to the Hindenberg explosion to a rhythmic rattle of metallic percussion for the airship's construction. The section about Dolly has biologists, artificial-intelligence researchers and ethicists commenting on cloning and identity. The emphasis of this movement is reflected by a statement by Richard Dawkins that: "We, and all other animals, are machines created by our genes". Dawkins' impersonal visage uttering "machines" is seized on and looped seemingly endlessly.

This is genuine, compelling art that attracts lively and diverse audiences. It is a unique and morally ambiguous response to challenges whose origins lie in science. But it is built, in part, on soundbites that amount to a provocatively simplistic summary of what modern biology is telling us. Nevertheless, for those intrigued by a face-to-face encounter between outstanding music theatre and the implications of twentieth-century technology, this is a must-hear, must-see. ■





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Healthy outlook
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'Mile-deep club' of researchers sets sights on disused gold mine

Geoff Brumfiel, Washington

Plans for a new National Underground Science Laboratory in an abandoned gold mine in South Dakota have now gained the backing of both physicists and geologists. But just as scientific interest in the Homestake mine gathers pace, political momentum is waning.

Physicists have already chosen the Homestake mine as their first choice for the new lab (see *Nature* **410**, 292; 2001), on the grounds that it is deeper than the alternatives and so is better protected from cosmic rays, which can interfere with their experiments.

At a National Science Foundation (NSF) meeting, held in Washington on 19–21 September to discuss plans for an underground lab, geologists also gave Homestake their backing. They say that the 2,500-metre-deep mine is ideal for studying geology, hydrology and subterranean bacteria.

For physicists, the site offers a chance to conduct studies such as the Underground Nucleon Decay and Neutrino Observatory — a 400,000-tonne water tank designed to detect neutrinos and proton decays. Observing such a decay would provide evidence for theories of supersymmetry, which could help unify grav-



Going underground: Brookhaven National Laboratory's solar neutrino experiment at Homestake.

ity with the three other fundamental forces. Some physics experiments, such as the neutrino studies run by the Brookhaven National Laboratory, already take place at Homestake.

Around 70 of the 300 delegates at last week's Washington meeting were geologists.

One reason for their support is that Homestake offers the potential to study subterranean bacteria that can live for hundreds of thousands of years, says Tullis Onstott, a geomicrobiologist at Princeton University. These bacteria feed on minerals rather than organic

Polar project confirms suspicions about early Universe

Alison Abbott

A faint signal from the primordial Universe has provided an important confirmation of theories that describe the period immediately after the Big Bang.

Researchers from the University of Chicago, speaking at the COSMO-02 workshop on particle physics and the early Universe, held last week in Chicago, presented the first evidence of a tiny polarization in the cosmic microwave background radiation. Such a polarization had been predicted by standard inflationary theories, which hold that the Universe underwent a rapid expansion immediately after the Big Bang. "This confirms, almost beyond doubt, that we really have understood the very young Universe," says Simon White, a director of the Max Planck Institute

for Astrophysics in Garching, Germany.

The cosmic microwave background dates from some 300,000 years after the Big Bang, at which time the Universe was cool enough for protons and electrons to combine to form atoms. This allowed photons to travel freely through the Universe, as atoms interact less frequently with photons than free protons or electrons do. These photons form the microwave background.

One key prediction about the microwave background — that its temperature varies in different parts of the sky — has already been confirmed. The temperature differences are thought to be the remnant of small density fluctuations that seeded the formation of galaxies (see *Nature* **411**, 880–881; 2001).

Theory predicts that the density fluctuations will also have caused some

polarization of the microwave photons — a prediction now confirmed by the Chicago team. They studied 200 days' worth of data taken from two points in the sky by the National Science Foundation's Degree Angular Scale Interferometer radio telescope at the Amundsen-Scott South Pole Station. The effect is slight and has eluded scientists for decades, but the telescope was sensitive enough to identify a polarization that matches the predictions.

Researchers now want to increase the sensitivity of the polarization measurements. Several teams are now focused on this goal, including the consortium that runs Balloon Observations of Millimetric Extragalactic Radiation and Geophysics (BOOMERANG) — a balloon-borne telescope that navigates Antarctica. ■

BROOKHAVEN NATL LAB

compounds, and so could provide clues about early life on Earth. Little is known about them because scientists are rarely able to study them at the temperatures and pressures at which they naturally live.

"There are many questions that need to be answered," says John Parkes, a geo-microbiologist at the University of Bristol, UK. "A deep mine will be very valuable."

Both Republicans and Democrats had originally been keen to back the project in order to win support for their candidates contesting the South Dakota seat in this November's Senate elections.

But with political enthusiasm lessening as attention turns to Iraq, a dispute between the NSF, which is expected to fund and run the lab, and Barrick Gold Corporation, Homestake's owners, could scupper the plans of physicists and geologists. The NSF is adamant that the proposal must undergo peer review, but in a public letter last month, Barrick's vice-president Patrick Garver stated that the process would take too long. "It appears to us that a final determination is unlikely to be finalized for several years," he said.

Moreover, Congress is reluctant to agree to Barrick's demand that the NSF assumes environmental liability for the lab before the takeover. If an agreement cannot be reached soon, Barrick says it will allow the mine to flood.

Nonetheless, scientists at the Washington conference remained hopeful that Homestake, or perhaps another site, will become a reality. They say they appreciated the chance to discuss one another's fields, and look forward to the possibility of sharing a lab. "To have these kinds of conversations around the lunch table would just be fantastic," says Onstott. ■

BSE in human tissue fires debate on patient disclosure

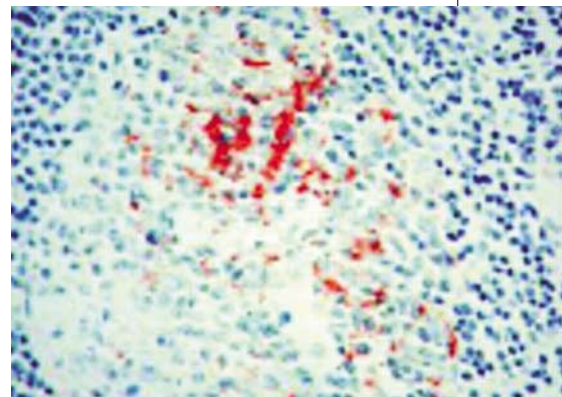
Erika Check

For the first time, a tissue sample removed in Britain during routine surgery has tested positive for the human form of bovine spongiform encephalopathy (BSE). The find has reopened the debate over whether people found to be incubating the fatal neurodegenerative disease should be informed.

Last week, researchers announced that they had turned up one positive sample of the misshapen protein, or prion, thought to cause variant Creutzfeldt-Jakob disease (vCJD), among 8,318 tonsils and appendices removed from patients in the late 1990s (D. A. Hilton *et al. Br. Med. J.* **325**, 633–634; 2002). Earlier data from 3,000 samples had returned negative results (see *Nature* **405**, 7; 2000).

From this, the researchers estimate that about 120 out of every million Britons might eventually develop vCJD, thought to be the result of eating meat products from cattle infected with BSE. That number falls within previous estimates, based on epidemiological modelling, of several thousand cases. But, given that the new calculations are based on just one case, the margin of error remains large. "We're not going to jump to any conclusions," says David Hilton of the Derriford Hospital in Plymouth, who led the team.

Hilton says that testing of further samples under the existing study, in collaboration with the CJD Surveillance Centre in Edinburgh, will continue through next year. And the British government's chief medical officer, Liam Donaldson, adds that a larger tissue-screening study is planned, which may further



Tell-tale tinge: a red colour betrays the presence of BSE prion in this human appendix sample.

clarify the disease's eventual prevalence.

Meanwhile, Swiss scientists have begun their own screen of tissues from surgery and autopsies. Adriano Aguzzi, a neuropathologist at the University Hospital in Zurich, says doctors will examine thousands of samples over the next decade or so. But whereas the UK survey is anonymous, so that patients who test positive cannot be notified, the Swiss survey will retain patients' details.

"We think the science in the prion field is advancing very quickly, and it's not unlikely that in three or five years' time there will be a way to block the prion from causing disease," says Aguzzi. If therapy becomes available, Swiss test results will be made available; they will also be disclosed to specific patients who request them even in the absence of a cure. ■

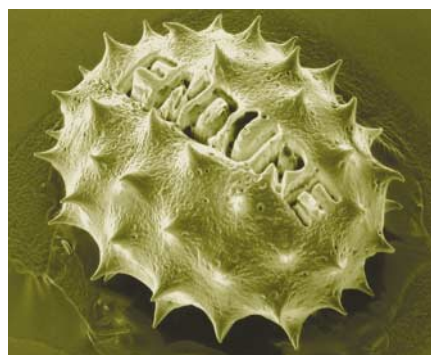
D. A. HILTON ET AL. *BR. MED. J.* **325**, 633–634 (2002)

Nanoscale etchings let art lovers read the small print

Carina Dennis, Sydney

For Stephanie Valentin, an artist at Sydney's College of Fine Arts, it was a new world to explore. Paul Munroe, a materials scientist at the University of New South Wales in Sydney, found it a rare opportunity to apply a tool commonly used for studying electronic circuits to a biological specimen. The result — a series of micrometre-high words carved into grains of pollen — went on display in Sydney's Stills Gallery on 11 September.

Valentin approached Munroe in her quest to find a method for writing on pollen. He suggested using a focused ion beam miller: a device usually used to etch the surface of electronic circuits. With Munroe's help, Valentin used a narrow beam of gallium ions to carve words on



Not to be sneezed at: one of the pollen grains that have been transformed into works of art.

pollen grains with a diameter of around 20 micrometres.

The project has useful scientific spin-

offs, as other researchers are interested in using focused ion beam millers to study biological samples. "What is notable is that they were able to use it to etch pollen without destroying it in the process, and with a very high degree of precision and control," says Ian Williams of the Australian National University (ANU) in Canberra, who uses ion beams to analyse geological samples.

"Not much work has been done on biological materials," says Sally Stowe, also at ANU. Stowe plans to use focused ion beams to scrutinize the interface between hard and soft materials, such as titanium implants in bone. The ion beam should allow her to carefully cut away layers of the sample without damaging it. "It's a nanoscale Swiss Army knife," she says. ■

S. VALENTIN

Hostilities resume over future of GM crops

David Adam, London

Like two punch-drunk boxers preparing for one last bout, the two sides in the British debate over genetically modified (GM) crops are getting ready to clamber back into the ring. A raft of consultation exercises and debates on the controversial technology have been launched in recent weeks, as industry groups and environmental activists prepare for the government decision on whether to allow commercial planting of the crops, which could be made next year.

The new efforts centre on a £250,000 (US\$390,000) scheme to foster debate on transgenic crops, paid for and promoted by the Department for Environment, Food and Rural Affairs. Those planning the exercise held their first meeting in London on 13 September. The debate's format has not been finalized, but one suggestion is to make and distribute a short film featuring the various viewpoints and to follow it up with a series of local discussion meetings. The results will be handed to the government next spring.

Lobby groups are already trading punches. On 17 September, the Soil Association, an organization that promotes organic farming, published a report suggesting that transgenic crops had cost the US economy \$12 billion since 1999. The report cited a combination of low prices and falling exports to countries that are reluctant to buy food products containing GM ingredients. Product recalls, such as that required when a brand of taco shells was found to contain transgenic StarLink maize, which is intended for animal consumption only, also played a role. GM proponents then highlighted a report, issued in June by the National Center for Food and Agricultural Policy, a Washington-based independent think-tank, which claimed transgenic crops had increased US farm income by almost \$1 billion in 2001, thanks to greater yields and reduced herbicide use.

Attention will soon focus on scientific studies of the crops. The first data from field-scale trials to assess the crops' impact on biodiversity are due early next year (see *Nature* 412, 760–763; 2001). But senior scientists say the studies are too limited to judge whether or not the new technology should be adopted. The trials do not address important aspects of the debate, including economic issues and the possible flow of transgenes into conventional crops.

The British government has ordered a review of issues such as gene flow and a study into the costs and benefits of GM crops. It has acknowledged that it is under pressure from US biotech companies to approve the crops, but has promised to consider the results of the new studies and the consultation exercise before making any decision.



Passing economic and environmental tests may not be enough, however. The European Union is adamant that any food products containing transgenic ingredients should be clearly labelled as such, and this may deter consumers. Daniel Pearson, secretary of the Supply Chain Initiative on

Modified Agricultural Crops, a pro-GM industry group, says British farmers are interested in using the technology, but he admits that consumer reluctance could be a problem. "Clearly, farmers won't grow something they can't sell and you can't prejudice that they would find a market for it," he says.

The debate has also thrown up some unlikely alliances, not least that between the Natural Environment Research Council, Britain's main environmental research funding agency, and *spiked*, an online publication set up from the ashes of the now-defunct *Living Marxism* magazine. The council is sponsoring a debate about the field-scale trials on the *spiked* website — a decision that has already brought complaints from researchers.

"It is an unusual partnership," admits Marion O'Sullivan, a council spokeswoman. "But there's something about the GM debate that draws these comments. Nobody said anything when we sponsored one on climate change." ■

Italy's space partners left in the dark

Sally Goodman

The man at the head of Italy's national space agency, ASI, is ruffling feathers both at home and abroad.

Sergio Vetrella's plan for the agency's 2.8-million-euro (US\$2.8-million) budget for 2003–2005 leaves the future of many projects in doubt. The biggest single category in the budget for 2005 is "initiatives yet to be approved", leaving it unclear whether funding for other areas, including collaborations with NASA and the European Space Agency (ESA), remains intact.

The plan was approved by the Italian government on 5 August, but the details will not be finalized until the national budget is set later this year.

ASI's partners have already asked for clarification. David Southwood, director of ESA's science programme, says he is expecting an answer before his 15 October deadline about ASI's commitment to Venus Express, an exploratory mission to Venus planned for 2005 (see *Nature* 418, 360; 2002). "I will have little choice but to cancel the mission if they do not come on board," he says.

NASA is waiting to hear whether Italy will go ahead with building a water-seeking radar for the Mars Reconnaissance Orbiter, which is due to launch in 2005. "We will need a definite commitment from Italy within the next couple of months," says a NASA spokesman.



Grounded: Sergio Vetrella's plans for the space agency leave collaborations in doubt.

Vetrella, who was appointed last November, says that many project proposals were in a confused state with no clear written objectives when he arrived. "There was no time to include them in the defined part of the space plan. I preferred to separate them from the more mature projects," he says.

Southwood has also asked for assurances that the Italian instrument for Planck, ESA's 2007 mission to observe the cosmic microwave background, will be delivered on schedule. But he adds that Planck is suffering because other countries are also behind schedule for payload construction, and that in the future he wants member countries to make firmer commitments before industry is engaged. "There have been too many gentleman's agreements," says Southwood. ■

Satellite-image users fear private price hike

Tony Reichhardt, Washington

It didn't work then, and it may not work now. That's the message from some global-change researchers, who fear that privatization of the Landsat satellite system could drive up the price of images of the Earth's surface, just as it did when the service was taken out of public hands in the 1980s.

Landsat satellites have photographed the Earth for 30 years, providing researchers with a valuable record of changes such as deforestation. The current satellite — the seventh in the series — is operated by NASA and the US Geological Survey, but Congress has repeatedly asked for its successor to be privatized. A draft invitation for bids to run the system is expected from NASA in the next few weeks, with the formal version due by the end of the year.

The successful company would have to build and launch a replacement for Landsat 7, which is designed to last at least until 2004, but is expected to operate for several years beyond that. In return, the firm would be paid by the US government to provide a regular supply of images of the Earth. Satellite-imaging companies DigitalGlobe and Resource21, both based in Denver, Colorado, are currently working with NASA on the future of Landsat, and are expected to bid for the contract.

Landsat was privatized in 1985, but a commercial market for its pictures failed to

develop. Left to market forces, researchers ended up paying thousands of dollars for a single image. This inhibited wider scientific use of remote-sensing imagery, according to a 1997 National Academy of Sciences study, and was the reason that the government resumed control of Landsat in 1992. Costs have remained at a subsidized rate of \$600 per image since Landsat 7's launch in 1999.

The new Landsat contract will require the operator to supply the government with images of 30-metre resolution. Remote-sensing companies warn that there is insufficient commercial demand for such images, so the winning company may choose to use technology that can produce images of 10-metre resolution. These would be of interest to agricultural companies, as they can, for example, be used to monitor crops. But it is unclear whether this extra market will be enough to make a commercial Landsat profitable.

Without additional business, many Landsat users worry that the contractor will resort to high prices as a way to make ends meet. Ray Williamson, a remote-sensing policy expert at George Washington University in Washington DC, doubts that a commercial vendor can beat the current price of Landsat 7 images, and says that commercial clients are right to be concerned about the future.

If privatization fails again, some fear that



Eye in the sky: Landsat images, such as this one of sand dunes in the Namib Desert, are a rich source of data for global-change researchers.

LANDSAT

the government could abandon its investment in Landsat altogether. Samuel Goward, a remote-sensing expert at the University of Maryland in College Park and chair of the Landsat science team, says that Congress has been very clear that it wants the next satellite to be commercially owned. "If there isn't a business involvement, the mission itself may disappear," he says.

Ocean geologists hatch plan to probe ancient zone

David Cyranoski, Tokyo

US and Japanese geologists are planning a major investigation of the Izu-Bonin-Mariana Arc, a 2,800-kilometre subduction zone located in the Pacific Ocean south of Japan. Studying the region, where one oceanic plate is sliding underneath another, could shed light on many geological processes, including how the first continents formed. "This collaboration will be one of the most concentrated geoscientific efforts over the next ten years," says Jim Gill, a geologist at the University of California, Santa Cruz.

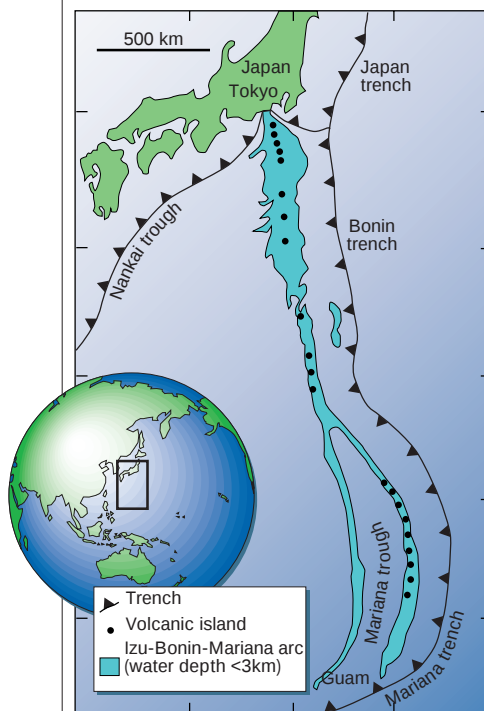
Japanese researchers will have racked up seven one-month research missions to the area by the end of the year, and the US National Science Foundation (NSF) has invested \$4.5 million in studies there over the past few years. At a meeting held in Hawaii earlier this month by the NSF and Japan's Institute for Frontier Research on Earth Evolution, researchers outlined their plans for the coming decade.

Studies of the arc have been prioritized by the NSF and various Japanese research institutes, partly because the crust over

oceanic plates is thinner than the continental crust, making it easier to obtain information from seismological signals. The relatively old age of the plate, which has been undergoing subduction for 43 million years, is a further incentive. Some researchers believe that the first continents were created in similar oceanic subduction zones. "It is the closest thing to how our continents got started some 3–4 billion years ago," says Gill.

At the Hawaii meeting, experiments to be conducted with *Chikyu*, a new ocean-drilling ship that is due to enter operation in 2006 (see *Nature* 415, 356; 2002), were cited as central to future studies. Geologists want to use the vessel's 7-kilometre drilling capacity to sample directly the composition of the crust and underlying mantle.

Time on *Chikyu* will be in short supply, however, as other groups are competing for time on the vessel. A single drilling mission takes around a year, so trips will be limited. A smaller collaboration is also bidding to use *Chikyu* to explore a subduction zone under the Aleutian Islands off the coast of Alaska.



NIH head looks to the 'biomedical century'

Erika Check, Washington

The first time Elias Zerhouni heard from a US president was in 1985. Ronald Reagan was suffering from colon cancer and Zerhouni, a radiologist at Johns Hopkins University School of Medicine in Baltimore, Maryland, had just pioneered a new technique for differentiating between benign and cancerous growths. Reagan's doctors got in touch: would Zerhouni be willing to help treat the president using his new technique?

Zerhouni answered the president's call, just as he did again earlier this year when George W. Bush asked him to take over at the National Institutes of Health (NIH) in Bethesda, Maryland. Zerhouni was sworn in as the NIH's fifteenth director four months ago, and a photo of Reagan hangs behind his desk. Last week, he began to discuss his plans for the "biomedical century"—from the need for the NIH to sell itself, to how he will use the people skills he honed as an immigrant to advance the agency.

Zerhouni finds himself at the helm of the NIH just as the agency reaches the end of a five-year doubling of its budget, which should take next year's funding to \$27.3 billion. But the cash influx has brought problems. Some observers fear that funding will stagnate once the present increase is complete. Others worry that the agency's decentralized structure prevents it from funding cutting-edge research. Congress has appointed a committee to look at how the NIH is organized, and some panel members say that reorganizing the agency's 27 separate institutes into clusters would be more efficient (see *Nature* **418**, 572; 2002).

Speed trials

Trumpeting the NIH's successes is one way to answer critics, says Zerhouni. He points out that the agency began investing in research into West Nile virus when it was first discovered in New York in 1999. Three years later, clinical trials of a candidate vaccine are under way. "Does that say this is an organization that's sclerosed and not flexible? I don't think so," he says.

This and other success stories could help convince Congress to keep on increasing NIH funding, but Zerhouni admits that the agency doesn't always do a good job of explaining why investment in biomedical research is useful. Asked how to make this case to the public, he pulls out a thick book of health statistics and traces the rise in deaths due to human immunodeficiency virus (HIV) through the 1980s to a peak in the mid-1990s. Without federal investment in the science behind retroviral therapies, he says, this line would have marched on and HIV would now kill at least eight times as many people in the United States as it



Appointed director by George W. Bush earlier this year (left), Elias Zerhouni wants to manage change at the NIH while publicizing its triumphs.



does. "If this is not getting a return on your money, I don't know what is," he says.

Nonetheless, Zerhouni realizes that the agency may have to change. New areas such as imaging, nanotechnology and proteomics require input from a range of disciplines. It's a process that he is familiar with, having assembled a multidisciplinary team of scientists to develop new imaging technologies when he was at Johns Hopkins—but not one that is suited to the NIH's structure. "The fundamental question is, can we execute strategies that are beyond the mission of one institute and yet are the responsibility of the entire NIH?" he asks.

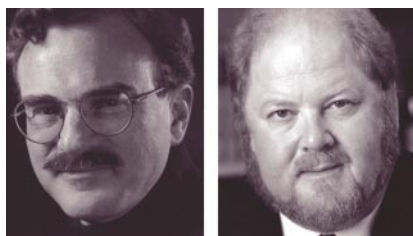
Zerhouni also has a big recruitment job on his hands. Several institutes currently lack directors, including the National Institute of General Medical Sciences and the National Institute of Neurological Disorders and

Stroke. He has already named new leaders at the National Institute of Mental Health and the National Institute on Alcohol Abuse and Alcoholism (see *Nature* **419**, 240; 2002), and says that filling the remaining posts is one of his top priorities. "I take a lot of pains in recruiting people," Zerhouni says, adding that his job has been made somewhat easier by the recent increase in the NIH's budget. "Everybody feels that this is a historic moment in biomedical research."

It's easy to see how Zerhouni's calm, friendly manner could win the confidence of a prospective employee. Even as he ponders the big picture, he tries to make personal connections. Zerhouni arrived at his interview with *Nature* fresh from sharing a platform with the surgeon general, Richard Carmona. "I always look at people and say there must be a contact point," he says. He hadn't found one before with Carmona, but that day he had: Carmona grew up in Spanish Harlem, the New York City neighbourhood where Zerhouni's daughter, Yasmin, now teaches at a public school.

Zerhouni says he developed his skills at dealing with people as an entrepreneur during the early 1980s. He founded five companies, although he ended all connection with them on taking up his new post. Arriving as an immigrant from Algeria at the age of 24 also helped, as he had to create a new network of colleagues and friends. But his daughter keeps him humble, he says. "She tells me there's a greater need for teachers than there is for NIH directors." ■

KENNETH LAMBERT/AP



Prize guys: Randy Schekman (left) and James Rothman worked on vesicle budding and fusion.

Cell biologists scoop Lasker prize for studies of vesicles

New York Two scientists who worked out the molecular mechanisms that enable cells to confine specialized functions within discrete cellular compartments have been honoured with this year's Albert Lasker Award for Basic Medical Research.

Over the past quarter-century, James Rothman of New York's Sloan-Kettering Institute and Randy Schekman of the University of California, Berkeley, have identified molecular components of the machinery that allows the budding and fusion of membrane-bound vesicles. These processes are vital for the formation of the cell's organelles, and for the movement of biochemical cargoes within and between cells.

♦ www.laskerfoundation.org

West Nile virus may spread through blood transfusions

San Francisco US health officials have confirmed that West Nile virus can survive in donated blood, and say it is likely that at least one person has contracted encephalitis through a transfusion.

Following suspicions that the virus had tainted the blood supply (see *Nature* 419, 102; 2002), officials at the Centers for Disease Control and Prevention in Atlanta, Georgia, are investigating six possible cases of virus transmission by blood transfusion. In one case, investigators cultured the virus from frozen plasma. The same donor's blood had been given to a patient in Mississippi who developed West Nile encephalitis.

Animal-rights group buys chimps' freedom

San Diego A chimpanzee research centre that became a *cause célèbre* for the animal-rights movement has been purchased by a group that plans to retire the facility's 266 chimps and 61 monkeys.

The White Sands Research Center in Alamogordo, New Mexico, operated by the Coulston Foundation, was sold on 13 September to the Center for Captive Chimpanzee Care in Fort Pierce, Florida.

Many of the animals were used in research into hepatitis B and HIV. They became a focus for protest after the Coulston Foundation was cited for breaches of federal animal-welfare regulations.

Captive Chimpanzee Care was able to purchase the White Sands centre thanks to a \$3.7-million donation from the Arcus Foundation in Kalamazoo, Michigan, which promotes gay and animal rights.

Japanese court dims LED researcher's patent hopes

Tokyo Japanese researchers are slowly waking up to the profitability of holding patents. But in a setback for scientists demanding more credit and financial gain, one of the country's most famous inventors has lost the first round of a legal battle against his former employer.

Shuji Nakamura, now at the University of California, Santa Barbara, was fêted for his work on blue light-emitting diodes (LEDs), carried out while he was working at Nichia Corporation of Tokushima. But last year Nakamura, who worked for Nichia until 1999, sued the company for ¥2 billion (US\$16.2 million), which he claimed he was owed as the inventor (see *Nature* 412, 844; 2001).

Under Japanese law, inventors are supposed to have control of patents. But the Tokyo district court ruled that Nakamura handed over the 1991 blue-LED patent to Nichia under the company's internal rules. The court will now consider Nakamura's claim that Nichia failed to give him "reasonable compensation".

Rice biologists steam ahead with gene tools

Beijing Two new information resources have been made available to rice researchers and farmers following announcements made at the International Rice Congress in Beijing last

week. The International Rice Research Institute (IRRI) near Manila, the Philippines, officially opened its Rice Knowledge Bank, a freely available database of information and tips on topics such as pest management and the translocation of hybrid rice. IRRI hopes that the project will close the gap between lab research and agricultural practice.

At the same meeting, the Beijing Genomics Institute, which has sequenced the *indica* subspecies of rice, *Oryza sativa* (J. Yu *et al.* *Science* 296, 79–92; 2002), said that it will supply microarrays containing 54,000 predicted genes to IRRI and Yale University in New Haven, Connecticut. The analysis will help researchers locate genes for crucial traits such as pest resistance.

♦ www.knowledgebank.irri.org

Syngenta uproots deal with UK plant-science centre

London The multinational agribusiness Syngenta has cancelled a £50-million (US\$78-million) ten-year research partnership with the John Innes Centre in Norwich, UK, as part of its restructuring plans.

In 1998 the John Innes Centre signed the deal with the British company Zeneca. In return for exclusive access to aspects of the centre's work on wheat genomics, Zeneca agreed to bankroll a new laboratory and pay for other research at the centre and at the nearby Sainsbury Laboratory.

Syngenta, formed in 2000 by the merger of the agribusiness arms of Zeneca and Novartis of Basel, Switzerland, inherited the John Innes deal. But last week it announced its decision to withdraw from the alliance. John Innes officials say the deal's demise was not a surprise — but it is still a major blow. "Transferring what we learn to industry is a key part of the research process, and this decision is obviously a setback to this process," says Chris Lamb, the centre's director.

Dutch government cuts science budget

The Hague The Netherlands' new conservative government last week slashed almost 8% off the 2003 budget for science and technology, dashing researchers' hopes of boosting the country's expenditure — which is below the European average as a proportion of national wealth.

The previous Liberal–Socialist coalition, voted out this summer, had promised to boost Dutch science spending by some 250 million euros (US\$245 million), 100 million euros of which was earmarked for the Netherlands Organization for Scientific Research (NWO).

"The cuts will increase above tolerable levels the already excessive competition for grants," says Peter Nijkamp, president of the NWO, which supports basic research.



Top tips: the Rice Knowledge Bank is set to make lab research more useful to rice farmers.

Sitting in judgement

Investigations of scientific misconduct need expert input, but they can prove harrowing experiences for the scientists involved. Erika Check finds out why.

When Jeffrey Flier picked up the telephone on a January day 22 years ago, he was surprised to hear his old supervisor on the end of the line. Jesse Roth was director of the lab at the National Institutes of Health (NIH) in Bethesda, Maryland, where Flier had worked as a postdoc, and he had a favour to ask. One of Roth's postdocs had accused a Yale University postdoc of plagiarism — would Flier investigate?

"I tried to talk myself out of it," says Flier, now at Harvard Medical School. At the time, there were no rules for conducting such investigations. Also, the accused postdoc worked for Philip Felig, one of the most highly regarded researchers in Flier's field of metabolic disease. Flier eventually relented, but found the experience unsettling. He uncovered serious fraud, which ended the career of the postdoc, Vijay Soman, in the United States and derailed Felig's plans to take up a departmental chair at Columbia University in New York. "I would never go into it by myself again," he says.

Thankfully, things have improved since then. In the United States, investigators of misconduct in biomedicine now work in teams and are backed up by federally funded experts. But despite all this, many researchers finish inquiries feeling much as Flier did. Some have faced lawsuits from the accused. Others have had to end a fellow scientist's career. And all have to juggle the inquiry with their day-to-day academic lives, even though

It's like being dragged off the street and told to function as a policeman.

Paul Friedman

cases can drag on for years. "It's very unpleasant to have to judge your colleagues," says Paul Friedman, emeritus professor of radiology and former dean of academic affairs at the University of California at San Diego. "It's like being dragged off the street and told to function as a policeman for the next week."

Attitudes to misconduct investigations vary between disciplines. High-profile physics cases at Bell Laboratories in Murray Hill, New Jersey, and the University of California at Berkeley have hogged headlines this year (see *Nature* **418**, 5; 2002 and *Nature* **418**, 261; 2002). But misconduct is probably more common in biomedicine, where competition and commercial pay-offs are often greater.

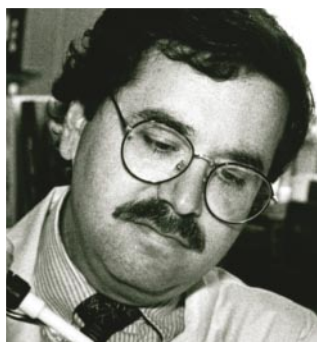
National approaches also differ. Most European countries have no central organization for dealing with research misconduct, and investigations are handled by institutions or funding agencies. The United States, however, has a dedicated organization for monitoring misconduct, for biomedicine at least. Now known as the Office of Research Integrity (ORI), this body was established in 1989 and

is currently run by the US government's Department of Health and Human Services. ORI says that 127 new misconduct allegations were made in 2001, the third consecutive rise since 1998. Most dealt with accusations of fabrication, such as inventing patients who didn't exist, or falsification, where existing data were distorted (see chart, opposite).

Under ORI rules, every institution that receives federal grants for biomedical research has to have a formal procedure for investigating misconduct. In many centres, new cases are handled by an in-house research integrity officer — a former scientist or, in the case of Margaret Dale at Harvard Medical School, a lawyer. When Harvard investigates a misconduct allegation, Dale starts by working the telephones. She forms a committee of three senior scientists from an appropriate field, avoiding those who work with the accused. "It should be someone who will be comfortable making an unpopular decision, and who's not going to be running into this person in the cafeteria or on other hospital committees," she says.

Dale says that researchers are generally happy to be involved as long they have the time and are not connected with the scientists involved. But once the committee begins the sometimes laborious process of interviewing the accused and any witnesses, as well as combing through relevant data, people often start to have second thoughts.

The time required is one of the biggest



Reluctant heroes: (left–right) Jeffrey Flier, William Chambers, Paul Friedman and Margaret Dale.

problems. ORI says that the length of an investigation varies greatly, but that researchers are involved for an average of nine months. Those taking part soon realize that the work will distract from their research without adding to their own reputation or publications list. “They gain almost nothing from it,” says Friedman. “Mostly they just make enemies.”

The investigation of cancer researchers Friedhelm Herrmann and Marion Brach, Germany’s most high-profile misconduct enquiry (see *Nature* **418**, 113; 2002), is a case in point. Ulf Rapp, the University of Würzburg cell biologist who led the investigation, says he could not have refused when he was asked to look into the matter in 1998. But he had no idea that the enquiry would last three years. “I would have limited my involvement to the first year,” says Rapp. “We thought we would work for a while and then transfer our findings to a different committee.”

When an investigation finally comes to an end, researchers must deal with the results — a difficult task, as a guilty verdict can stop a promising career dead in its tracks. William Chambers, an immunologist at the University of Pittsburgh, has served on several misconduct panels. Last year, he completed an enquiry into a Pittsburgh molecular biologist who said that his data related to different experiments from those he had actually performed. “It was easy to see, although he didn’t admit it,” Chambers says. As a result, the post-doc — a foreign national — left both the university and the country. “I still feel a little sad about it,” Chambers says. “This was someone who could have had a bright future.”

Other researchers finished their investiga-

tions only to find themselves the subject of a lawsuit. Susan Berget, a geneticist at Baylor College of Medicine in Houston, Texas, led a 30-month inquiry into her colleague Kimon Angelides, who was accused of falsifying data in five papers and in NIH grant applications worth \$4 million. Angelides, who studied sodium channels in nerve cells, was sacked from Baylor in 1995 after being found guilty both by Berget’s team and then by a federal appeals committee. But he went down fighting, slapping slander lawsuits on Berget and other investigators, as well as on the college.

Angelides settled the case in 1999 after an NIH investigation confirmed Berget’s findings (see *Nature* **397**, 549; 1999). But Baylor College was landed with a legal bill of more than \$2 million, and Berget was left wishing she had never got involved. “If someone had told me up front what this would take out of my life, I would have run away,” she said at the time. She now declines to discuss the case for fear of being sued again.

Berget is not the only scientist to harbour regret long after the event. “My name got in the news a lot, but it wasn’t the kind of news I was looking to be associated with,” Flier says of his inquiry. “Over the years this thing keeps coming up, and not everyone can keep straight who was investigating and who was the investigator. I’ve had to defend myself against the view that I was the perpetrator.”

Despite the stress involved, current procedures have the backing of biomedical researchers. It may be a distraction from everyday duties, but scientists say there is no better way to separate real fraud from a baseless accusation than to have researchers exam-

ine the facts. “You need to get people who are involved in research so they know how the experiment is done,” says Chambers. And while the accused has to suffer a long trial, the procedure is now similar at most institutions, and allows for several levels of appeal.

Ideas for improving the process are thin on the ground, however. Lawsuits are impossible to avoid altogether, although cases such as Berget’s could be made less difficult if institutions briefed investigators about what legal steps the party under investigation could take, and what protection investigators have. Most institutions, for example, have insurance policies that cover legal costs.

Reducing the time involved may be less easy. David Wright, assistant vice-president for research ethics and standards at Michigan State University in East Lansing, has one alternative. If members of the panels he oversees do a good job, he writes a letter to the dean or chairperson of their department and requests that their work be taken account of in their salary review — although it is unclear whether such requests can compensate researchers for the time they spend on an investigation.

Other researchers have raised the idea of appointing full-time committees to look into misconduct allegations. The idea has little support, however, as most scientists would prefer investigators to be actively involved in related research. “If you don’t enforce your own standards you don’t have the moral authority to set them,” says Friedman.

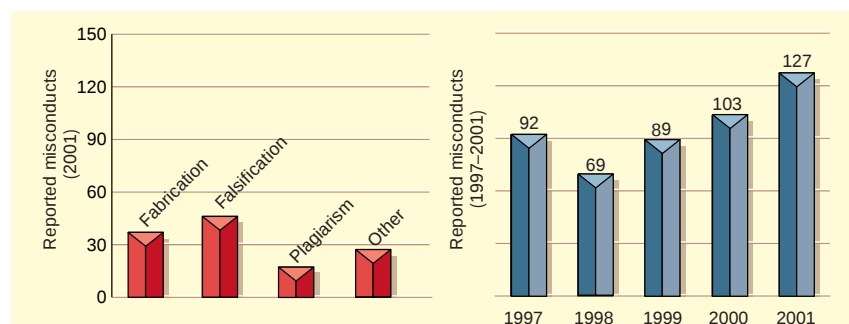
With radical changes to the investigative process in short supply, the most practical approach may be to tackle the root of the problem — the fraud itself. No single person can end the competitive pressures of biomedical research, but, says Friedman, they can combat fraud by stopping minor infractions before they reach the level of serious misconduct. “Nobody arrives at fraud as the first thing they ever do,” he says. “That’s important. They got there by doing little things and getting away with it. Calling them in might stop people from going off the deep end.” ■

Erika Check is *Nature*’s Washington biomedical correspondent.

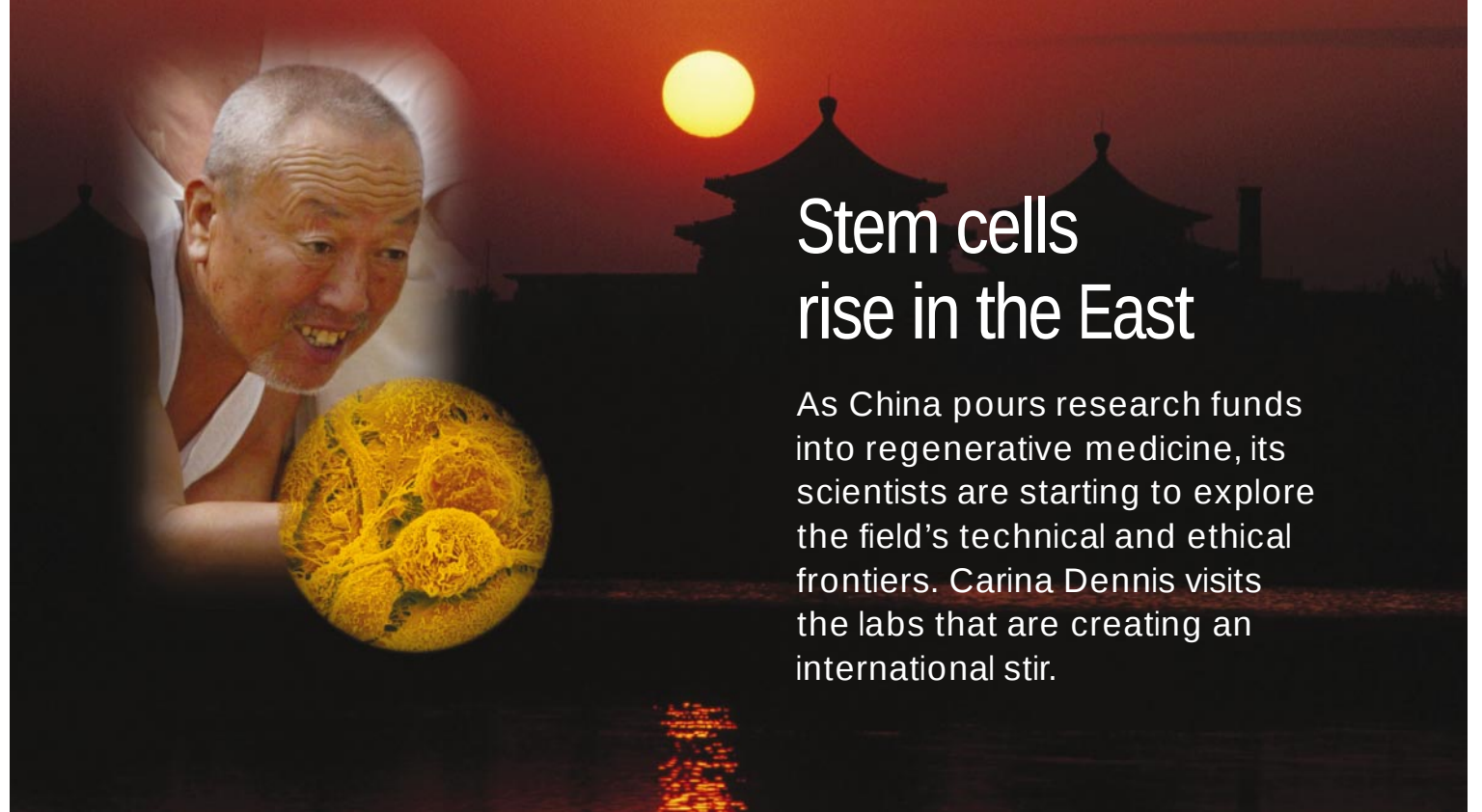
► <http://ori.dhhs.gov>

LEFT TO RIGHT: BETH ISRAEL DEACONESS MEDICAL CENTER; L. D. GREGORY; SCRIPPS INST. OCEANOGRAPHY; S. GILBERT

ORI



Figuring it out: allegations of misconduct reported to the Office of Research Integrity in recent years.



Stem cells rise in the East

As China pours research funds into regenerative medicine, its scientists are starting to explore the field's technical and ethical frontiers. Carina Dennis visits the labs that are creating an international stir.

*"When joy is at its highest
Sad thoughts run rife
Youth and strength, how short they last
How hopelessly we age!"*

Emperor Han Wudi, Western Han Dynasty¹

More than two millennia ago, China's emperors searched in vain for the elixir of youth. Today, facing the demographic consequence of its one-child policy — a steadily ageing population — the country as a whole is grappling with the same challenge.

Rather than turning to wistful poetry, China's current leaders see a solution in the field of regenerative medicine. They have started to spend lavishly on labs working on stem-cell research and therapeutic cloning, hoping to develop the means to grow cells and tissues to replace those lost to the ravages of age and degenerative disease.

With this generous support, and in a cultural environment in which there are fewer of the moral objections to the use of embryonic stem cells that have hampered the field's development in the United States, Germany and elsewhere, expatriate Chinese biologists have rushed back home. "There has been a real boom in stem-cell research in China in the past two years," says Lingsong Li of Peking University in Beijing, who returned in 2000 from a postdoctoral position at Stanford University in California.

Questions remain about China's ability to attain a world-class presence in the field. But as news about the projects under way in China circulates through the scientific community, and reports of the most eye-catching work emerge in the international press,

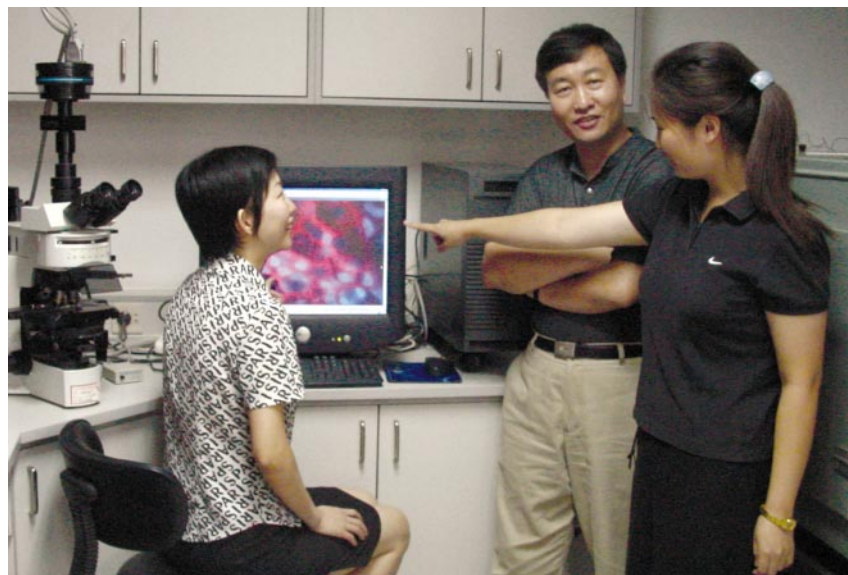
Western researchers are taking notice. "It is clear that there is a group of scientists doing good work," says Peter Donovan, a stem-cell biologist at Thomas Jefferson University in Philadelphia, who was a keynote speaker at the 2002 International Symposium on Stem Cell Research, held in Beijing in May. And *Nature* recently had a rare opportunity to visit some of their labs.

A few hours spent with Li provide some intriguing insights into China's current stem-cell push. He has the confident air of a Californian biotech entrepreneur — and the research facilities to match. Li heads the Peking University Stem Cell Research

Eyes on the prize: will stem cells help maintain the health of China's greying population?

Center, which boasts a staff of some 50 people. He has received more than US\$4 million — from the university, the central government, and Chinese venture capitalists — to set up the centre and to cover its running costs for three years.

As he shows visitors around the gleaming new labs, Li fields calls on his mobile phone from his scientific, political and business contacts. Energetic and ambitious, he is attracted to the fast pace of stem-cell research — and to China as the right place to pursue it. His rise



Booming business: Lingsong Li (centre) enjoys the fast pace of emerging stem-cell research in China.

to prominence has certainly been meteoric. Li does not have an established track record in the field, but says that his training at several US universities, mostly in immunology, gave him the confidence to seize his current opportunity. "I am motivated by the challenge of growing within the field, as well as growing the field within China," says Li.

His centre will focus on how the differentiation of embryonic stem cells is regulated, and will try to apply various types of stem cells to the treatment of cardiovascular disease, neurological disorders and diabetes. Li's group has developed a method of genetically altering neural stem cells so that they can multiply indefinitely in culture but retain their ability to differentiate into neurons. His team has also created animal models of human neurological diseases to study the signals that tell stem cells how to develop and direct them to sites of injury where they are most needed.

Creating a buzz

Li may be one of the main movers and shakers on China's stem-cell scene, but the most intense international interest has so far focused on the leaders of two other labs. In March, with rumours about the work already buzzing around the stem-cell research community, *The Wall Street Journal* reported that a team led by Guangxiu Lu of the Xiangya Medical College in Changsha in central China had since 1999 been creating cloned human embryos with the goal of extracting stem cells. The same article claimed that researchers led by Huizhen Sheng of Shanghai Second Medical University had extracted stem cells from embryos created by fusing adult human skin cells with rabbit eggs that had been stripped of their own chromosomes.

Sheng's work is based on the now-familiar concept of therapeutic cloning — cloning an embryo from the patient's own cells to harvest stem cells that could be used to grow tissues genetically matched to the patient, so eliminating problems with immune rejection. Sheng turned to rabbit eggs because the human variety is in short supply. Human eggs would be used if therapeutic cloning ever becomes a clinical reality, but the idea is to develop the techniques involved without exploiting this precious resource.

Other researchers are working along similar lines — the biotech firm Advanced Cell Technology (ACT) of Worcester, Massachusetts, revealed in 1998 that it had experimented with cloning using human cells and cow eggs. But ACT is not thought to have extracted stem cells from the resulting embryos. The report of Sheng's research also came at the height of the US political debate over cloning, and was seized on by lobbyists on both sides. Those in favour of therapeutic cloning argued that the United States risked losing the lead in an important field, whereas anti-cloning activists sought



Huizhen Sheng (left) has trained a large team of young researchers to work on embryonic stem cells.



to portray China as a morally bankrupt 'Wild East' of biology.

Unlike ACT, which has been widely criticized for making media announcements about unpublished work of uncertain scientific merit, Sheng did not court publicity. *The Wall Street Journal* picked up the story from someone who had heard Sheng present her data at the University of Texas Southwestern Medical Center in Dallas. Disturbed by the attention focused on work that has not been peer-reviewed, she has no intention of discussing its details until it appears in the scientific literature. "We receive hundreds of calls from journalists," says Sheng. "I don't blame the media — they are doing their job. But if data are reported too early, it can interrupt the normal process of scientific review."

Sheng's lab is an oasis of calm in the hub-bub of central Shanghai — mirroring her circumspection amid the media scrum that has surrounded her work. Situated on the top floor of a building in the Xinhua Hospital, her lab overlooks the city's rooftops. In her bright, airy office, our conversation is punctuated by the faint sounds of sirens as ambulances pull up to the emergency department downstairs.

Along the corridor, students are peering down microscopes at tiny balls of embryonic cells dividing in culture. Sheng heads a team of over 40 people, more than half of them students, and she thrives in her role as teacher and mentor. But it was tough to get the lab started, because so many of China's top young scientists go abroad. "I had to

Searching for an ethical consensus

Mention the phrase 'embryonic stem cells' in many Western countries, and you're thrown headlong into arguments over the morality of extracting cells from human embryos just a few days old. But in China, the potential medical benefits are seen as justifying the practice. "The population more readily accepts this research," says Renbiao Chen, a member of the Chinese National Human Genome Center at Shanghai's bioethics committee.

Cynics might argue that China lacks the democratic traditions likely to foster a public debate on the issue. But Chinese researchers and officials are anxious to dispel the notion that the country is an unregulated frontier for stem-cell science — not least because they want to attract investment and scientific collaborators from the West. "We need to set up guidelines that follow international standards," says Lingsong Li, who heads the Peking University Stem Cell Research Center in Beijing.

Draft national regulations on stem-cell research are due later this year, and are expected to borrow from Britain's liberal legislation. If so, Chinese researchers would be able to extract new human embryonic stem-cell lines. Harvesting stem cells from embryos created by cloning may also be allowed.

While officials in Beijing wrestle with the details, scientists in China's second city have drawn up their own rules. Stem-cell researchers in Shanghai already have to comply with guidelines drafted by the bioethics committee at the city's genome centre, which were published in October last year in China's leading medical ethics journal⁵. The Shanghai guidelines are also based on Britain's regulations, with one significant difference — they allow experiments that aim to harvest stem cells from embryos created by fusing human cells with eggs from other mammalian species that have been stripped of their own genetic material.

This is only allowed for basic research — clinical therapeutic cloning would have to use human eggs. But it is unclear whether the national regulations will follow Shanghai's permissive lead. "People in the scientific community and government agencies have different opinions," says Li.

recruit fresh students and train them for a couple of years," she says.

Sheng returned to China in June 1999 after spending seven years as a postdoc with Heiner Westphal, a developmental biologist at the National Institute of Child Health and Human Development in Bethesda, Maryland. There, she had become interested in the possibility of using nuclear-transfer cloning to reprogramme adult cells to an embryonic state. However, constraints on the use of US federal funds would have prevented her from pursuing the research in Bethesda. "At that time, the Shanghai municipal government offered me a large grant to set up a lab," says Sheng.

But this opportunity came at a personal price. "My husband loves his job in the United States and my son is now at college," says Sheng. Although she sees them several times a year, the separation is hard to bear. "I miss them very much, but it is such an exciting time in science and that keeps me going."

Ethical dilemma

Sheng remains troubled by criticisms of the ethics of her work. Far from operating in an ethical vacuum, she points out that all research on embryonic stem cells in Shanghai adheres to guidelines published last year (see 'Searching for an ethical consensus', previous page). "I think people have a tendency to think that scientists in China just push their research wherever they want it to go. But this is not the case," she says.

Although many Western experts have become sceptical about the prospects of therapeutic cloning ever being made efficient enough to become a routine clinical procedure, there is more enthusiasm in China. At least one other Chinese group in addition to those of Lu and Sheng — that of Xigu Chen of Sun Yat-Sen University of Medical Sciences in Guangzhou — has also experimented with the technique².

Whether China can make a clinical success of therapeutic cloning and other stem-cell therapies won't be clear for some years. The strategy has been to concentrate funding in about a dozen laboratories. "Research labs can find themselves better off than those in the West," says Robert Chunhua Zhao, executive director of the National Key Laboratory for Experimental Hematology in Tianjin, southeast of Beijing, and head of the National Center for Stem Cell Research, which opened in the same city this year.

China has tried the strategy of investing heavily in a select few centres in other areas of biology. Since 1998, it has launched a handful of DNA sequencing facilities, which participated in the international Human Genome Project³. Yet despite an increasing number of publications in top-tier journals, China's accomplishments in the wider field of genetics remain modest compared with those of leading scientific nations.



While cities such as Shanghai prosper, many Chinese are too poor to benefit from medical advances.

Opinion is divided on whether the prospects are brighter in regenerative medicine. "The gap between stem-cell research in China and other countries is not so great," says haematologist Xuetao Pei, director of the Beijing Institute of Transfusion Medicine and Stem Cell Center. But bringing stem-cell therapies to fruition will require expertise in a wide variety of disciplines, from molecular, cellular and developmental biology, through immunology and tissue culture, to clinical medicine. And in that regard, the relatively low level of China's research base could pose problems. Working in one of the country's new stem-cell labs is a little "like doing research on an island", concedes Pei.

Share the knowledge

Vigorous networking between Chinese stem-cell researchers and colleagues working in the West might help. "It is important that we share information to address the imbalance," says Linzhao Cheng, who works on stem-cell engineering at Johns Hopkins University in Baltimore, Maryland.

But even if such links succeed in providing China's stem-cell push with the necessary foundation in basic biology, findings must still be transferred from the lab to the clinic. "I'm not convinced that the interdisciplinary medical teams needed exist in China," says Edward Lin, a clinical oncologist at the M. D. Anderson Cancer Center in Houston, Texas, who regularly visits the country.

Zhao, who returned to China in 1999, disputes this. He spent several years in the lab of Catherine Verfaillie at the University of Minnesota in Minneapolis — who works on 'mesenchymal' stem cells in adult bone marrow that, under some conditions, can differentiate into a variety of tissues⁴. Now Zhao is trying to coax mesenchymal stem cells to differentiate into blood cells, and he points to

his own national haematology laboratory, which has 350 beds for patients with blood diseases and has already conducted clinical trials of bone-marrow transplants. He has applied to initiate clinical trials using stem cells in China next year, transplanting mesenchymal stem cells into patients with chronic myeloid leukaemia. Pei also hopes to begin trials with the same cells, to treat disorders including liver and nervous-system diseases.

Alex Zhang, who returned from Stanford last year to head the Cell Therapy Center at Xuan Wu Hospital in Beijing, is similarly enthused by the clinical opportunities in China. But he is concerned that things may proceed too quickly. "I worry that doctors in China are too eager to get into clinical trials," says Zhang. "I would like to see some guidelines for stem-cell trials in place to avoid rushing into the clinic before the basic science has been done." The government is now working on such guidelines, but they are not expected to be in place for a year or two.

Although Zhao and others are confident that China has the necessary clinical expertise, they become less assured when asked about who is likely to benefit from stem-cell therapies. "One obstacle is the expense of the treatment," says Zhao. China may have a large and ageing population, but most are poor. Even if China's stem-cell pioneers achieve their scientific goals, sheer cost could mean that the therapies they develop become primarily a commodity for export — and for the new élite who have inherited the mantle of China's ancient emperors. ■

Carina Dennis is *Nature's* Australasian correspondent.

1. Watson, B. *The Columbia Book of Chinese Poetry* (Columbia Univ. Press, New York, 1984).
2. Abbott, A. & Cyranoski, D. *Nature* **413**, 339 (2001).
3. Cyranoski, D. *Nature* **410**, 10–12 (2001).
4. Jiang, Y. et al. *Nature* **418**, 41–49 (2002).
5. Committee on Bioethics of the Chinese Natl Hum. Genome Cen. *Shanghai Zhongguo yixue lunlunxue* **6**, 8–9 (2001).

Tension arises from duality at the heart of taxonomy

Names must both represent a volatile hypothesis and provide a key to lasting information.

Sir — H. C. J. Godfray (*Nature* **417**, 17–19; 2002) points out in his Commentary, quite rightly, that taxonomy and systematics, the naming and classification of living things, have an image problem among funding bodies and the community in general. He says the discipline faces a crisis: much information on living things needs to be captured, organized and disseminated, but funds often go into higher-profile projects such as the Sloan Digital Sky Survey and the Human Genome Project.

What do these projects offer that taxonomy does not? One thing is a relatively centralized model of data organization and retrieval. One can enter a web portal and easily find and download an authoritative account of star positions or gene sequences. No equivalent portals currently exist for taxonomic information (although initiatives are in place to provide them: see *Nature* **418**, 362–363 & 367; 2002). To create such a portal, Godfray suggests that taxonomy needs to move from the current dispersed, decentralized approach to a unitary, centralized one.

Although we agree that Godfray's model would provide many advantages to outside users, we believe that it misses one crucial problem. A taxon — the base unit

of a taxonomy — is an hypothesis, not an observation or “fact”. It is not like a star or a base pair, and taxonomy is not like a star survey or genome project. Because of this, solutions cannot be so easily transferred from these domains to taxonomy.

Taxonomists routinely filter large arrays of observations through an extensive knowledge base to decide that this group of specimens or that set of prior taxa comprises a new taxon. Crucially, another taxonomist using the same knowledge base may validly arrive at quite different conclusions, and it may take time for thorough testing of the alternative concepts to arrive at consensus (itself subject to future challenge and refinement).

Taxonomy is a complex and dynamic science, not a simple (albeit technically difficult) collation of facts like the nucleotide sequences of a genome survey. It is probably only at the most complex levels of genetics, in dynamic proteomics or developmental biology, that anything like the complexity of hypothesis-generation and testing involved in routine taxonomy and systematics is encountered.

Any proposal for changing the

processes of taxonomy must account for this. Godfray's model, in which new names require validation by a central committee before acceptance, could lead to authoritarianism and a stifling of innovative taxonomic viewpoints. No other hypothesis-driven field of science would accept such a straitjacket.

The heart of the problem is the central duality and tension in taxonomic names. On the one hand, names are convenient shorthand representations of scientific hypotheses, and as such should be as volatile as hypotheses in any other field — proposed, used, modified and then perhaps discarded as evidence dictates. On the other hand, taxonomic names are keys to biological information, unique codes that unlock the library of knowledge about those organisms, and for this purpose they must be non-volatile or the links will be lost. A solution must adequately recognize these dual roles and decouple the system that allows maximum freedom of hypothesis-generation from the system that provides names for users.

Kevin Thiele, David Yeates

CSIRO Plant Industry and Entomology,
Commonwealth Scientific and Industrial Research
Organisation, Canberra, Australia

No alternative to animal tests for behaviour

Sir — Your News story “Race is on to find alternative to animal tests” (*Nature* **418**, 116; 2002) discussed the possibility that *in vitro* methods could replace animal tests used in the toxicologic screening of chemicals. However, it is highly unlikely that *in vitro* approaches could be used to replace *in vivo* animal tests in the assessment of neurotoxicity. For instance, behavioural assessment in rodents is a powerful experimental tool in the evaluation of the effects of chemicals on neural function. Pharmaceutical companies and regulatory agencies consider behavioural toxicology an important part of the screening of new pharmaceutical products and potentially neurotoxic chemicals.

It is true that *in vitro* experimental approaches can be used in the investigation of the cellular and molecular mechanisms of behaviour. But drug-induced alterations in parameters such as anxiety, motor activity, and learning can only be assessed by *in vivo* tests on mammals. It is not possible to replace

such tests with non-animal methods using, for example, human cells or yeast.

Rafael Roesler

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Laws stay constant but the world changes

Sir — Efforts by Stephen Schneider and Richard Moss to quantify uncertainty (*Nature* **418**, 476–478; 2002) succeeded with the Intergovernmental Panel on Climate Change (IPCC) Working Group 1 climate modellers but failed with Working Group 3's emissions modellers. As a lead author on the IPCC's 2000 *Special Report on Emissions Scenarios*, I would like to elaborate on why this happened.

The first group of scientists know that the laws of physics operative today will be the same laws of physics operative in 2100. The second group, of economists and analysts, know with equal certainty that the geopolitical, socioeconomic and technological forces operative today are likely to be radically different in 2100.

Stuart R. Gaffin

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Food labels should state the benefits of GMOs

Sir — You state in the News story “Europe gets tough on labelling genetically modified foodstuffs” (*Nature* **418**, 114; 2002) that an angry food industry claims that stricter labels would imply a health risk.

On the contrary. The GMOs potentially present in trace amounts have been far more thoroughly assessed for safety than the rest of the food, but the labelling tells us nothing of this. The European Union has selected GMOs for special labelling because many people are uncertain of the benefits and risks. Surely, then, labels should convey the positive (and, where appropriate, negative) environmental, nutritional or economic attributes of the modification? This would help consumers make informed decisions to buy or avoid products.

Renton Righelato

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When fish learned to walk

The process by which fish took to the land occurred in several steps.

Gaining Ground: The Origin and Evolution of Tetrapods

by Jennifer A. Clack

Indiana University Press: 2002. 369 pp.

\$49.95, £37.95

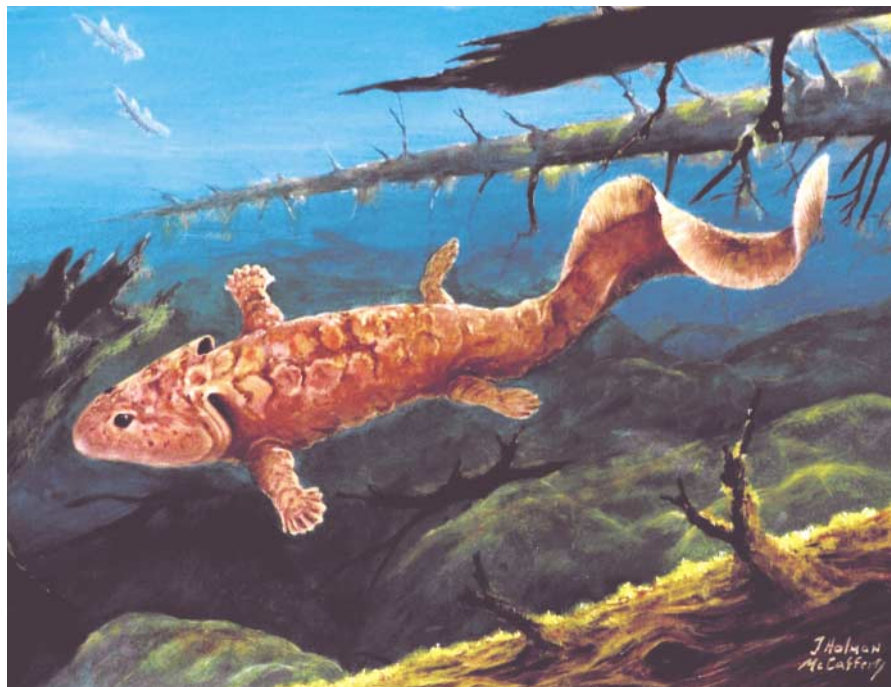
Philippe Janvier

The evolutionary transition from fish to air-breathing, four-legged land vertebrates, or tetrapods, is a fascinating segment of vertebrate evolution. It is increasingly well documented by fossils dating from the Late Devonian and Early Carboniferous periods, from 370 million to 320 million years ago. During the past ten years, a generation of young palaeontologists has discovered far more remains of these earliest tetrapods than ever before, and Jennifer Clack's earlier researches have been pivotal in this quest for the 'four-legged fish'. Her new book, *Gaining Ground*, presents data that have barely been dealt with before, even in the most recent textbooks on early vertebrates.

A major turning point in Clack's work occurred in the mid-1980s, with her incidental discovery of new skeletons of the Late Devonian tetrapod *Acanthostega*. These had been collected by geologists at the same Greenland site as *Ichthyostega*, long considered to be the key to the fish–tetrapod transition. Clack was also blessed with an outstanding preparator and several brilliant disciples, and her small team soon made the University of Cambridge a leading research centre in this field.

Thanks to their thorough study of *Acanthostega*, Clack and her collaborators discovered several apparently trivial anatomical features that turned out to be diagnostic for early tetrapods. This triggered a large-scale re-examination of new and old material from various Late Devonian localities in northern Europe. Some of these finds had been dismissed as "undetermined fish fragments" but turned out to be tetrapod remains, yet older and presumably more primitive than *Ichthyostega* and *Acanthostega*. The discovery of the key for recognizing tetrapod remains pushed back the earliest tetrapod evidence from 365 million to about 370 million years ago. By reviewing this new material and discussing its evolutionary significance, *Gaining Ground* is a particularly important update.

People often think that the most important event in the rise of land vertebrates occurred in the Late Devonian, when fins evolved into limbs. The main message of this book is that the fish–tetrapod transition and the conquest of land were quite separate evolutionary steps. Clack shows here that all



Landing gear: the early tetrapod *Acanthostega* had limbs with digits but still lived in the water.

Devonian tetrapods were essentially aquatic, with fish-like gills, and were merely lobe-finned fishes with strange, paddle-like paired fins elongated by new structures: the digits. The transformation of the paired fins into limbs does not imply walking or a terrestrial life, which probably occurred later, in the Carboniferous. As Clack puts it: "The problem actually consists of three parts: origin of limbs with digits, origin of walking, and origin of terrestriality."

Gaining Ground begins with an introduction to fish and tetrapod anatomy, the diversity and relationships of living and fossil osteichthyans (bony fishes and tetrapods), and the Late Devonian environments that prevailed during the fish–tetrapod transition. The description of the Devonian tetrapods known to date is followed by a review of all the anatomical changes that occurred at the fish–tetrapod transition, either documented by fossils or inferred from indirect evidence, and a consideration of the processes that may have been involved in this drastic change. In particular, Clack examines the latest data from developmental genetics of limbs and fins, which now appear to be relatively consistent with the story told by the fossils.

The second part of the book focuses on the Carboniferous tetrapods, a subject that has also been enriched by the discovery of many new fossils during the past ten years. Until recently, hardly any tetrapods were

known from the Early Carboniferous. This 20-million-year gap, known as Romer's gap, is now being filled by some recent discoveries (see *Nature* **418**, 72–76; 2002). These show that mobile wrist and ankle joints had appeared by the Early Carboniferous and that these tetrapods, although still confined to water, were capable of walking. This review of the Carboniferous tetrapods ends with a section on the relationships of the Palaeozoic tetrapods to the modern amphibians and amniotes. This section, which is limited to brief comments about the most recent cladistic analyses, is perhaps too succinct. It would have been interesting to know, for example, why the phylogeny proposed by Laurin and Reisz in *Amniote Origins: Completing the Transition to Land* (eds S. Sumida & K. L. M. Martin, Academic Press, 1997) is so radically at odds with all the others.

Gaining Ground ends with a review of all the character changes involved in the rise of terrestriality proper, in particular those in the ear, braincase, vertebral column and limbs. The various states of each major structure are presented in association with a cladogram, which aids their understanding.

Gaining Ground is an outstanding update of early tetrapod anatomy, phylogeny and systematics, with many interesting insights into the processes involved in the fish–tetrapod transition. Clack's pedagogic style and the numerous illustrations (which are generally clear and informative) mean that

it will be extremely useful to students and lecturers in palaeontology, geology, zoology and general biology, and is a 'must' for researchers in the field. ■

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Call of the wild

Signalers and Receivers: Mechanisms and Evolution of Arthropod Communication

by Michael D. Greenfield

Oxford University Press: 2002. 426 pp. £62.50, \$80

Ron Hoy

Communication between members of one's own kind is one of the most important behavioural acts that any animal must perform well if its genes are to be represented in the next generation. An alpha male (of any ilk) who has gained his 'top dog' advantage by virtue of his competitive prowess has fought for nought if his actions don't translate eventually into puppies of similar ilk.

This little book is ostensibly about how arthropods, but mainly insects, produce and consume signals in the behavioural commerce of competition and courtship. The evolutionary issues at stake, following Darwin, are neatly divided into the primary sensory modalities of sound, smell, sight and touch, and could as well be applied to fish, frogs, birds or mammals (and, if we follow the evolutionary psychologists, humans).

The book's subtitle pays homage to the fact that the diverse and numerous arthropods — most prominently, insects and spiders by land, and crabs and lobsters by sea — have some of the most varied modes of communication in the animal kingdom. Every possible sensory modality known (and some particular only to them) is exploited by arthropods for transmitting and receiving communication and signals. As a consequence, the topic of arthropod communication has generated a huge literature, and the book lists 71 pages of references, making it a valuable resource for this alone. The text itself is just 295 pages, but Michael Greenfield has done a thoughtful, excellent job of highlighting the central theoretical issues and selecting salient examples from this huge literature.

The chemical senses, smell and taste, are extremely important in arthropods and, depending on the species, mediate everything from finding food to finding a mate. Through hormonal actions they also mediate developmental decisions. The chemical senses seem to be crucial links in the behav-

ioural networks that enable the evolution of hierarchically complex societies of individuals. Greenfield discusses the behavioural and evolutionary aspects of olfaction admirably, primarily within the context of sexual advertisement and courtship, especially in moths, where much of the work has been done.

But in his coverage of moths, I wish that he had delved more deeply into the mechanistic aspects of communication, particularly its physiology and biochemistry, where so much excellent work has been done over the past 30 years. Thanks to work in selected model systems, such as the tobacco hornworm moth (*Manduca sexta*), we know a lot about molecular receptor systems, especially the neural systems that underlie developmental systems and courtship behaviour, which make up the neuroethology of communication. On the other hand, the virtues of this book are its conciseness and clear writing, so I suppose the author had to be selective about what to include.

The lion's share of attention is given to the chapter on mechanical signals, sound and vibration, which are the focus of the author's own research. From a comparative point of view, acoustic signalling is the province of only two groups of animals, the arthropods and the chordates. Greenfield devotes much of his narrative to the remarkably diverse ways and means by which insects produce and receive acoustic signals. Insects produce

signals by rubbing body parts together (stridulation), substrate vibration, tymbal mechanisms (in cicadas) and hissing; all are described, primarily in narrative form.

Here there appears to be a lost opportunity in the author's book plan. Although the book contains some excellent diagrams and other pictorial material, it could have done with at least twice as many depictions of mechanisms and even pictures of the animals themselves. Most of the illustrations are drawings, which is fortunate because the photographs are poorly reproduced. I was also surprised to see how little the author devotes to acoustic communication in the fruitfly *Drosophila*, given the status of *D. melanogaster* as a model organism, especially for behavioural-genetics studies.

The chapter on vision is the shortest but provides a sketchy outline on central issues of visual communication. Perhaps Greenfield was aware that the subject of arthropod vision has been masterfully covered in a recent book on the subject by Michael Land, *Animal Eyes* (Oxford University Press, 2001).

Signalers and Receivers concludes with a chapter on sexual selection and the evolution of signals, which is appropriate because insects have always been a rich source of insight into these topics. It is perhaps no accident that some of the major modern thinkers in evolutionary behaviour theory

Insects in focus

The Canadian painter-turned-photographer Jacques de Tonnancour captured these nocturnal wasps (*Apoica pallens*) on film in Brazil. At first (right, top) the wasps crawled all over their nest (top), but within 20 minutes, in a remarkable display of social behaviour, they had aligned themselves to protect the underside of their nest (bottom). De Tonnancour has spent the past 20 years photographing insects around the world. A collection of his work, with an insight into his subjects' ecology, anatomy and behaviour, can be found in *Insects Revealed: Monsters or Marvels?* (Cornell University Press, 2002), translated from the French by Luke Sandford.



were themselves entomologists, including W. D. Hamilton, R. D. Alexander and E. O. Wilson — so too was Alfred Kinsey, but that's another story. However, the five pages devoted to this chapter are so sketchy that I was left disappointed because insect studies have so much to bring to the general discussion. However, evolutionary issues arise and are discussed throughout the book.

In summary, this is a valuable addition to the literature on animal communication at an introductory level. Greenfield's book is worthwhile precisely because it is brief. I will recommend this book for undergraduate courses and for the generalist reader who wants to know more about this interesting subject. Advanced readers would also enjoy reading the book but may find it wanting for depth and detail, although they will greatly appreciate the reference list. ■

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Journey to the stars

Children of the Stars: Our Origin, Evolution and Destiny
by Daniel R. Altschuler
Cambridge University Press: 2002. 280 pp.
£19.95, \$28

Disturbing the Solar System: Impacts, Close Encounters, and Coming Attractions
by Alan E. Rubin
Princeton University Press: 2002. 376 pp.
\$29.95, £19.95

Charles A. Wood

Astronomy is the most awe-inspiring science. It seeks to understand the Universe: all time and matter, inconceivable distances, and ultimate questions of beginnings and endings, of life and existence. Generations of astronomers have written books to share the excitement of discoveries with the public, and the visually and scientifically stunning images from the Hubble Space Telescope and planetary exploration have generated a large number of recent popular books.

Scientists writing for the public have the difficult challenge of carefully explaining science yet maintaining a writing style that captivates lay readers. *Children of the Stars* and *Disturbing the Solar System* come close, but fall on either side of the ideal. They both range further than the Solar System, but this, as the human home, is their focus.

The perspective of *Children of the Stars* is spelled out by the title and subtitle. It is the now-familiar tale that humans are derived from stardust — a retelling and updating of popular books by George Gamow, Carl



Starstruck? Events such as the appearance of Comet Hale-Bopp fuel the public's interest in astronomy.

Sagan and others. Daniel R. Altschuler covers a lot of territory, including the size and structure of the Galaxy, how the Sun works, dying stars as creators of heavy elements, planetary formation, the geology of the Earth, the origin and working of life, giant impacts, and the human despoilation of our planet.

Altschuler brings a passion and attitude to his writing that makes *Children of the Stars* lively and personal. He delights in fascinating factoids (our brain contains as many neurons as there are stars in the Galaxy; there are more bacteria in your gut than there are humans on Earth), and provides humorous comments. Following his statement that some bacteria live happily in a polluted river, he adds: "Well, I have not actually asked them if they are happy." There is also a romantic speculativeness — for example, the 28-day human menstrual cycle may date from 500 million years ago when "biological clocks first might have been set in primitive living things" — that may make the reader question the scientific foundation of other statements.

Disturbing the Solar System is a very different book that covers many of the same topics. Whereas Altschuler often devotes only a paragraph or two to a subject, Rubin commonly provides a fuller history, including failures of understanding along the way. Most of the chapters are modest revisions of articles previously published in the *Griffith Observer* and other popular astronomy magazines. Each chapter ends with an obviously tacked-on transition paragraph to the next. The chapters have a well-defined focus, but also sometimes a pedantic completeness — such as listing all the moons of a planet — that fails to add to the story. Unlike Altschuler, Rubin is nearly invisible as an author: the word "I" occurs infrequently. The book is scientifically accurate, and the writing is quietly competent.

Rubin cannot be accused of merely

focusing on trendy, exciting topics. Nearly half of his chapters represent his research interests in meteorites, asteroids and impact cratering, which are important but not yet over-told tales. Two chapters on the Galaxy and ice ages seem out of place here. The book ends with the now *de rigueur* chapters on astrobiology, including a fascinating look at how humans might react if aliens were detected.

The books' dust-jackets suggest that these two books are aimed at the same general-public audience, but the books are quite different. Altschuler provides some thought-provoking one-liner titbits, while Rubin offers more thorough explanations. The level of science in Altschuler's book is similar to my son's eighth-grade textbooks, whereas Rubin's book approaches an introductory college text. Altschuler's book is a fun read, perhaps a transcript of well-received lectures he gives to the public; Rubin's volume could be used as a reference. The look and feel of the books reflect their writing styles. Most two-page spreads in Altschuler's book contain a colour photo (unfortunately with difficult-to-read blue captions), whereas Rubin's contains a much more limited number of mostly older, black-and-white photos, graphs and diagrams.

The science section of my local public library is overrun with glossy new books that sensationalize or water down science, throw-away volumes that end up a year or two later on the library sale tables. These two books rise above that level. Altschuler's *Children of the Stars* would be a good gift to excite anyone — nieces, nephews, parents and professionals in other fields — about the wondrous connections between humans and the cosmos. And for those who want more meat, Rubin's *Disturbing the Solar System* is hearty fare. ■

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Cells as computation

Aviv Regev and Ehud Shapiro

Although we are successfully consolidating our knowledge of the 'sequence' and 'structure' branches of molecular cell biology in an accessible manner, the mountains of knowledge about the function, activity and interaction of molecular systems in cells remain fragmented. Sequence and structure research use computers and computerized databases to share, compare, criticize and correct scientific knowledge, to reach a consensus quickly and effectively. Why can't the study of biomolecular systems make a similar computational leap? Both sequence and structure research have adopted good abstractions: 'DNA-as-string' (a mathematical string is a finite sequence of symbols) and 'protein-as-three-dimensional-labelled-graph', respectively. Biomolecular systems research has yet to find a similarly successful one.

The hallmark of scientific understanding is the reduction of a natural phenomenon to simpler units. Equally important understanding comes from finding the appropriate abstraction with which to distill an aspect of knowledge. An abstraction — a mapping from a real-world domain to a mathematical domain — highlights some essential properties while ignoring other, complicating, ones. For example, classical genetic analysis uses the 'gene-as-hereditary-unit' abstraction, ignoring the biochemical properties of genes as DNA sequences. A good scientific abstraction has four properties: it is relevant, capturing

an essential property of the phenomenon; computable, bringing to bear computational knowledge about the mathematical representation; understandable, offering a conceptual framework for thinking about the scientific domain; and extensible, allowing the capture of additional real properties in the same mathematical framework.

For example, the DNA-as-string abstraction is relevant in capturing the primary sequence of nucleotides without including higher- and lower-order biochemical properties; it allows the application of a battery of string algorithms, including probabilistic analysis using hidden Markov models, as well as enabling the practical development of databases and common repositories; it is understandable, in that a string over the alphabet A, T, C, G is a universal format for discussing and conveying genetic information; and extensible, enabling, for example, the addition of a fifth symbol denoting methylated cytosine.

We believe that computer science can provide the much-needed abstraction for biomolecular systems. Advanced computer science concepts are being used to investigate the 'molecule-as-computation' abstraction, in which a system of interacting molecular entities is described and modelled by a system of interacting computational entities. Abstract computer languages, such as Petrinets, Statecharts and the Pi-calculus, were developed for the specification and study of systems of interacting computations, yet are now being used to represent biomolecular systems, including regulatory, metabolic and signalling pathways, as well as multicellular processes such as immune responses. These languages enable simulation of the behaviour of biomolecular systems, as well as development of knowledge bases supporting qualitative and quantitative reasoning on these systems' properties.

Processes, the basic interacting computational entities of these languages, have an internal state and interaction capabilities. Process behaviour is governed by reaction rules specifying the response to an input message based on its content and the state of the process. The response can include state change, a change in interaction capabilities, and/or sending messages. Complex entities are described hierarchically — for example, if a and b are abstractions of two molecular domains of a single molecule, then (a parallel b) is an abstraction of the corresponding two-domain molecule. Similarly, if a and b are abstractions of the two possible behaviours of a molecule in one of two conformational states, depending on the ligand it binds, then (a choice b) is an abstraction of the molecule, with the choice between a and b determined by its interaction with a ligand process.

Cellular abstractions

Computer science could provide the abstraction needed for consolidating knowledge of biomolecular systems.

Using this abstraction opens up new possibilities for understanding molecular systems. For example, computer science distinguishes between two levels to describe a system's behaviour: implementation (how the system is built, say the wires in a circuit) and specification (what the system does, say an 'AND' logic gate). Once biological behaviour is abstracted as computational behaviour, implementation can be related to a real biological system, for example the detailed molecular machinery of a circadian clock, and the corresponding specification to its biological function, such as a 'black-box' abstract oscillator. Ascribing a biological function to a biomolecular system is thus no longer an informal process but an objective measure of the semantic equivalence between low-level and high-level computational descriptions. Equivalence between related implementations in different organisms can also be a measure of the behavioural similarity of entire systems, complementary to sequence and structure similarity.

Computer and biomolecular systems both start from a small set of elementary components from which, layer by layer, more complex entities are constructed with evermore sophisticated functions. Computers are networked to perform larger and larger computations; cells form multicellular organisms. All existing computers have an essentially similar core design and basic functions, but address a wide range of tasks. Similarly, all cells have a similar core design, yet can survive in radically different environments or fulfil widely differing functions. Of course, biomolecular systems exist independently of our awareness or understanding of them, whereas computer systems exist because we understand, design and build them. Nevertheless, the abstractions, tools and methods used to specify and study computer systems should illuminate our accumulated knowledge about biomolecular systems. ■

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FURTHER READING

► www.wisdom.weizmann.ac.il/~aviv

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Abstract work: a computer model of information transfer through a network of coloured points.



When good relationships go bad

David S. Hibbett

Mycorrhizal associations of fungi and plants are usually viewed as mutually beneficial, but some non-photosynthetic plants cheat their fungal partners. Molecular tools can now be used to identify the fungi being exploited.

Mycorrhizae are ancient, widespread associations between fungi and the roots of many species of plants. In these symbioses, the plants supply carbohydrates (the products of photosynthesis) to their fungal partners, which reciprocate by facilitating the uptake of mineral nutrients from the soil. In a reversal of the usual relationship, some non-photosynthetic plants — termed epiparasites — obtain carbohydrates from mycorrhizal fungi that are also associated with photosynthetic plants in the immediate environment. In other words, epiparasites feed off green plants in their communities, and they do so via a fungal 'bridge'.

A barrier to understanding the ecology of mycorrhizal plants, including epiparasites, has been the difficulty of identifying the fungi with which they are associated. There are two main types of mycorrhizae, ectomycorrhizae (ECM) and arbuscular mycorrhizae (AM). The first type is produced by mushroom-forming fungi — including choice edibles such as truffles and chanterelles. The second is produced by soil fungi that belong to the taxonomic order Glomales. These fungi produce no above-ground structures in their life cycle, and reproduction is accomplished by large, multinucleate spores that are produced underground and on which the taxonomy of the group has traditionally been based (Fig. 1a).

Until now, all documented cases of epiparasitism have involved ECM fungi. This is surprising, because AM symbioses involve roughly 70% of all plant species — including many agricultural crops — and are ecologically much more widespread than ECM symbioses, which involve only about 30 plant families, mostly trees. On page 389 of this issue, Bidartondo *et al.*¹ provide the first molecular evidence that the fungal partners of two distantly related groups of epiparasites are AM fungi.

Bidartondo *et al.* used the polymerase chain reaction to amplify fungal ribosomal genes (rDNA) from the roots of epiparasites in the Corsiaceae (a group related to lilies) and the Gentianaceae (gentian family). Anatomical studies in these groups had suggested that AM fungi are present, but gave no clues to their precise identity. Bidartondo *et al.* generated sequences of the fungal rDNAs and compared them to a database of

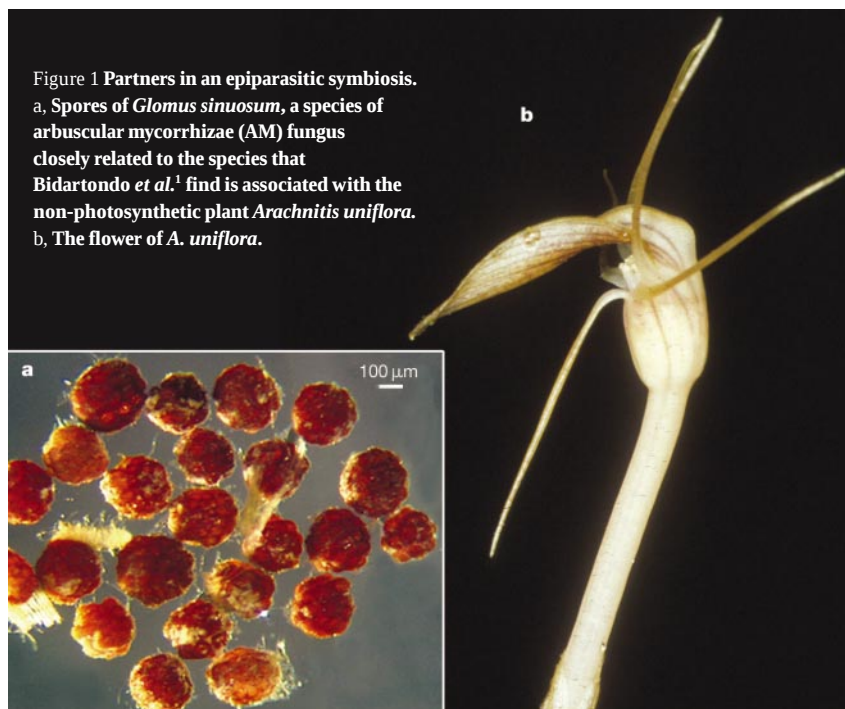


Figure 1 Partners in an epiparasitic symbiosis. **a**, Spores of *Glomus sinuosum*, a species of arbuscular mycorrhizae (AM) fungus closely related to the species that Bidartondo *et al.*¹ find is associated with the non-photosynthetic plant *Arachnitis uniflora*. **b**, The flower of *A. uniflora*.

Glomales rDNA sequences. Evolutionary analyses of the rDNA sequences provided the necessary taxonomic resolution, and showed that both groups of epiparasites are highly specific for particular species of Glomales.

In the most intensively sampled species, *Arachnitis uniflora* (Corsiaceae; Fig. 1b), Bidartondo *et al.* found that eight plants in three populations were all associated with a single fungal species. Neighbouring green plants harboured the same fungus in their roots, suggesting that they could be the ultimate source of carbohydrates for *Arachnitis*, although they also contained other AM species not found in the roots of *Arachnitis*. These findings mirror those in ECM-associated epiparasites^{2,3}, which also have very narrow host ranges. The results are of interest to evolutionary biologists in general, because they support the view that host specialization is a common consequence of the evolution of parasitic lifestyles.

Species of Glomales have previously been regarded as ecologically equivalent: that is, they do not differ appreciably in the range of plant species with which they are linked⁴. One reason for this view is that although there are thought to be as many as 300,000 species of plants that form AM associations,

there are only about 160 known species of Glomales. This is surely a gross underestimate of the actual diversity of Glomales, probably a consequence of their cryptic habit. Nevertheless, the striking disparity in apparent diversity of Glomales compared with that of AM-forming plants suggests that each species of Glomales must have many potential plant partners. The ecological-equivalence hypothesis is also supported by greenhouse studies involving pairings of individual species of fungi and plants, which have repeatedly shown that a single fungal species can form AM associations with many different plant species.

The results of Bidartondo *et al.* show that some kinds of AM associations are highly specific, and so contradict the idea that all species of Glomales are ecologically equivalent. The findings are less surprising in the context of studies indicating that, although species of Glomales are not absolutely host-specific, they exhibit ecological specialization under natural conditions. For example, the reproductive success of individual species of Glomales, measured in terms of spore production, varies according to the plant species with which they are associated^{5,6}. Conversely, in greenhouse studies simu-

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JOSEPH B. MORTON

lating natural plant communities, species of Glomales differ in their ability to promote growth of particular plant hosts⁷. The underlying mechanisms responsible for these differential growth effects are unknown. Bidartondo and colleagues' study¹ raises the tantalizing possibility that asymmetries in fungus-mediated transfer of carbohydrates between plants could be a factor.

The new work¹ is significant because it demonstrates that Glomales, which produce by far the most common form of mycorrhizae, can be drawn into epiparasitic associations. It also joins a growing body of research suggesting that mutualisms are not stable endpoints in evolution, but are inherently unstable and can be disrupted by conflicts of interest among the partners^{8,9}. The breakdown of mutualisms can lead to parasitism¹⁰ or even the complete dissolution of the symbiosis^{11,12}. In the groups studied by Bidartondo *et al.*, the plants are parasitic on AM fungi and their associated green plants. At the other extreme, an AM fungal species, *Glomus macrocarpum*, has been implicated as the cause of stunt disease of tobacco plants¹³.

Clearly, the characterization of AM symbioses as benign, stable associations does not

reflect their dynamic nature. Functional and ecological studies are now needed to quantify the costs and benefits of mycorrhizal symbioses and to understand what causes them to shift along the continuum from mutualism to parasitism¹⁴.

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reaction and adding as little as 1% chiral alcohol sealed the fate of the product.

Singleton and Vo¹ simply asked whether the nonlinearity of this reaction was sensitive enough to amplify the random, statistical imbalance that occurs naturally in a 'balanced' mixture of handed molecules. To their surprise, the products were formed in imbalanced proportions, but from reaction to reaction the amount of chiral product did not vary in a statistical way, indicating a systematic cause for the selectivity. Oddly enough, Soai had patented the same reaction, assuming random behaviour⁷.

Through a series of careful control and doping experiments, Singleton and Vo¹ deduced that chiral impurities in the reaction solvent — at the level of parts per billion and too small to be detected directly — were at the root of the phenomenon. Their results corroborate Soai's report of the effect of minor amounts of chiral additives on this reaction and emphasize the extreme sensitivity of the autocatalytic reaction. The high degree of selectivity and the apparent ability of so many different additives to instigate this selectivity demonstrate the need for additional experimentation and a complete kinetic analysis⁸.

The work highlights a caveat in the study of the origin of biological homochirality: the need to reconcile studies of a reaction that has a highly nonlinear response to deviations from the achiral state with the inherent presence of chiral impurities after a billion years of life on the planet. In contrast to the worries of biological contamination in a modern context, there remain issues of degradation or re-equilibration of the handed forms in a prebiotic context, which Singleton and Vo address.

Indeed, one problem associated with trying to explain the prebiotic origin of achiral symmetry breaking as being due to crystallization is that the system would re-equilibrate when crystals dissolved or degraded. But coupling chirality in crystals with chemistry as selective as the diisopropyl zinc/pyrimidine carboxaldehyde system means that symmetry breaking during crystallization could be propagated from one type of compound to another. Clearly, any process capable of shifting the balance in one setting need only leave a trace imbalance to influence the outcome of an autocatalytic reaction such as the one investigated by Soai.

Many proposals for the origin of biological homochirality have been predicated on the idea that a deterministic mechanism is necessary³ — that is, that the handedness we see in biomolecules must inherently be determined in the laws of physics, like the loss of parity. Singleton and Vo's study, in conjunction with Soai's work, supports the idea that abiological autocatalysis could be the process by which a randomly generated trace chiral influence was amplified. If so,

Chemistry

Shattered mirrors

Jay S. Siegel

How did the preference for 'single-handedness' in biological molecules arise? Amplification of the trace imbalance in a mixture of handed molecules bolsters the case for chance being the answer.

Some molecules are chiral — they exist in two forms that are mirror images of each other, right-handed and left-handed. Thus it seems reasonable that reactions that form chiral molecules from purely achiral precursors should produce equal amounts of each handed form to preserve achiral symmetry. So how did biological processes develop a preference for one or the other, such as left-handed amino acids, and right-handed sugars? In the *Journal of the American Chemical Society*, Singleton and Vo¹ report that, starting from two achiral compounds, they have prepared a chiral product in which one handed form dominates over the other. Have they demonstrated a fundamental principle that explains the mystery surrounding the predominance of a particular handedness in biomolecules?

The superstition surrounding the immutability of the achiral state has led to a reverence for molecular phenomena that break such reaction symmetry. However, for many years it has been known that such symmetry breaking can occur randomly through a variety of different mechanisms²: for exam-

ple, during the crystallization of a solution in which both hands are in equilibrium³, or in the spontaneous formation of chiral liquid-crystal domains⁴. In such cases, a small deviation from the achiral state is coupled to a process that is self-perpetuating and exponentially self-amplifying — that is, autocatalytic. As yet, spontaneous achiral symmetry breaking has not been observed for a reaction that occurs completely in homogeneous solution, and that is what Singleton and Vo were hoping to test.

The authors were following up the work of the Japanese chemist Kenso Soai, who reported an autocatalytic reaction in which a slight excess of one hand in the product suffices to direct any future product to that same handed form^{5,6}. The reaction scheme involved batchwise additions of diisopropyl zinc to 2-methylpyrimidine-5-carboxaldehyde, recycling the product as the catalyst for the next batch. The selectivity (that is, the fraction of handed product formed) was determined as a function of batch number. In Soai's experiments, numerous alcohols were claimed to influence the course of the

then in the prebiotic world examples of dominant molecular handedness were already likely to have been abundant. Thus, biomolecular handedness is an imperative of the evolutionary process downstream from molecular handedness, and smart money still bets on chance over determinism⁹.

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Membrane transport

The making of a vesicle

Anne A. Schmidt

The transport of molecules from one cellular compartment to another often requires membrane-bounded carriers. New work gives insight into how the shaping of membrane into such vesicles is linked to the selection of cargo.

If you could look inside your cells you would see several different compartments, delimited by membranes composed of lipids and proteins. You would notice that the compartments are highly dynamic and undergo a series of changes in morphology, allowing small, sack-like structures to bud off from time to time. These membrane-bounded carriers can differ in molecular composition, size and shape, and shuttle cargo from one compartment to another. One of the biggest challenges in cell biology is to unravel the molecular events that shape these vesicles emerging from a membrane, and that couple this process to the selection of cargo. On page 361 of this issue, Ford and co-workers¹ propose a mechanism of action for proteins known as epsins, and suggest that they bridge the gap between cargo selection and vesicle shaping.

Among the several types of vesicle that bud off from the plasma membrane — the membrane that surrounds the cell — some can be seen to be decorated by an electron-dense meshwork when visualized at high resolution, for example by electron microscopy. This meshwork is referred to as the membrane coat and is mainly involved in shaping the vesicle. It is often composed primarily of two protein complexes: clathrin and the AP2 adaptor complex. A whole bunch of accessory proteins are also part of the coat and control membrane budding, through molecular connections that involve protein–protein as well as protein–lipid interactions² (Fig. 1).

Proteins of the epsin family³, which are under the spotlight in Ford and colleagues' study¹, are among these accessory proteins. Epsins have at one end (the amino, or N, terminus) a structurally defined module that binds phosphoinositides — lipids bearing a head group composed of a so-called inositol

ring, modified with phosphate groups (phosphorylated) at several positions. This module, called the ENTH (for 'epsin N-terminal homology') domain, is also found in other proteins involved in membrane dynamics^{4,5}. Among these, the brain-specific protein AP180 and its ubiquitously expressed relative CALM are also implicated

in the formation of clathrin-coated vesicles.

Previous structural analyses^{6,7} of the ENTH domains of CALM and epsin, in the presence of the lipid phosphatidylinositol-4,5-bisphosphate (PIP₂), revealed a telling fact: these domains bind PIP₂ differently. In fact, the domain in AP180 and CALM is now referred to as the ANTH (for 'AP180-like N-terminal homology') domain. Ford *et al.*¹ now delve a little deeper. Their structural studies show that the PIP₂-binding site in the ENTH domain of most (but not all) epsin-family members involves positively charged amino acids that are dispersed through several of the structural units (α -helices) that build up the domain. Upon PIP₂ binding, the domain is reorganized into a deep basic groove in which the phosphorylated inositol head group is buried. This is radically different from the ANTH domains of AP180 and CALM, which bind PIP₂ through a small, positively charged stretch of consecutive amino acids⁶.

Ford *et al.* also find that this discrepancy in the lipid-bound structures of the ENTH and ANTH domains relates to functional specificity in vesicle budding. They show that epsin is recruited to artificial PIP₂-enriched liposomes (spherical structures that, like natural vesicles, are bounded by a lipid bilayer), and can bend this template into tubules. Amazingly, this also happens when the experiment is done with epsin's

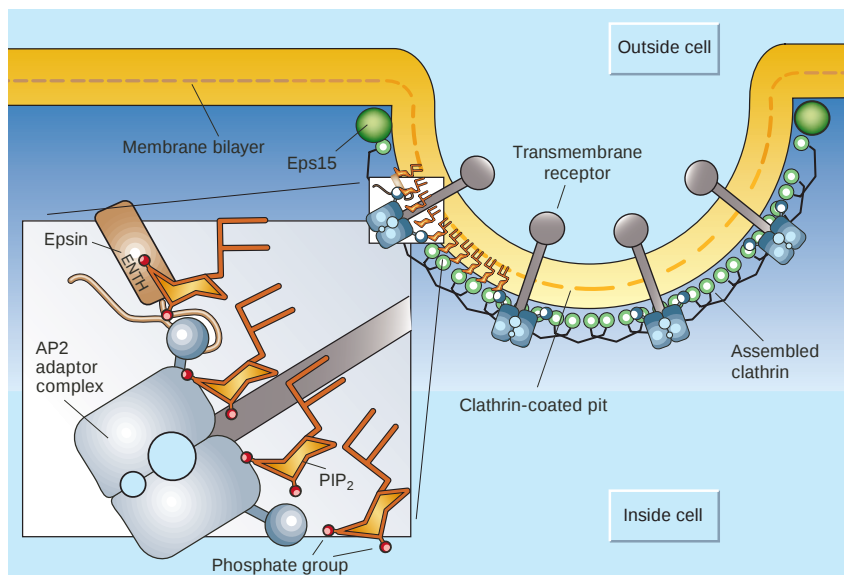


Figure 1 Bending membranes. A vesicle is shown budding from a cell's plasma membrane into the cell. Ford *et al.*¹ propose that epsin proteins induce the membrane curvature associated with budding, and couple it to loading of the vesicle with cargo. Epsin associates with clathrin and the AP2 adaptor complex, basic components of a budding vesicle's 'coat'. Epsin's ENTH domain also binds the phosphorylated head group of the lipid PIP₂ in the nearby leaflet of a membrane bilayer (see inset). Ford *et al.* propose that this disturbs the bilayer's stability, contributing to membrane curvature. Through its interaction with AP2, which in turn interacts with cargo receptors, epsin might couple initial membrane invagination to cargo recruitment. Then, while the coated pit is growing, epsin might affect membrane curvature at the rim of the coat, where another of its partners, Eps15, has been detected. Note that other proteins can also induce membrane curvature, and future work should reveal their spatial and temporal roles.

ENTH domain alone, hinting that the phenomenon is strictly induced by binding to PIP₂. But the ANTH domain does not cause membrane deformation.

So the authors propose that the burying of PIP₂ deep within epsin's ENTH domain encourages deformation of a membrane bilayer (Fig. 1). They suggest that this burial allows the ENTH domain to insert its N-terminal α -helix into the outer leaflet of the bilayer, pushing the lipid head groups apart. This would provide sufficient mechanical force to bend a flat membrane into an emerging bud. As further work showed that epsin binds to the AP2 adaptor and triggers clathrin-coat assembly, and that the combination of epsin and clathrin induces invagination of lipid monolayers, the authors propose a crucial role for epsin in vesicle budding. According to their model, epsin links its membrane-bending effect to cargo recruitment (through AP2, which binds the tails of transmembrane cargo receptors) and to coat assembly.

Epsin is not the only protein that can induce membrane curvature. At least three other proteins that are involved in clathrin-coated vesicle formation can trigger membrane tubulation independently *in vitro*. These proteins are dynamin, amphiphysin and endophilin^{8–10}. Why are so many actors playing what appears to be the same part?

It seems likely that these proteins have subtly different roles *in vivo*, which are difficult to detect in *in vitro* tests. For instance, for a long time clathrin was envisaged as being sufficient to drive membrane budding, given its *in vitro* ability to self-assemble spontaneously into a polyhedral structure resembling the one surrounding a coated vesicle. Nowadays, that view is being revised, on the basis of experiments and theoretical calculations¹¹, and it seems clear that several proteins must work together to bend a biological membrane. A challenge now is to determine whether these proteins induce membrane curvature *in vivo* as well as *in vitro* by binding to phospholipids.

It will also be important to find out when the proteins work — some might function during the initiation of budding, some later, and some at multiple stages. Although *in vitro* data show that all these proteins can initiate deformation from a flat membrane, suggesting that they take part in early events, this may result only from artificial capping of too many of the target lipids, because artificial templates are more enriched in these lipids than are biological membranes. This might be reinforced if the proteins insert significantly into the outer leaflet of a bilayer, inducing membrane bending by increasing the surface of the leaflet¹².

Of all the proteins that initiate membrane curvature *in vitro*, epsin might be the best candidate for this effect *in vivo*, given that its bending action may go hand in hand with

cargo recruitment — a process that begins when the membrane starts to curve. Nevertheless, it is plausible that some of these proteins induce membrane curvature in several different steps of budding. *In vivo* evidence indicates that endophilin might do so¹². Such could be the case for epsin, too. As well as working in the earliest steps of membrane curvature, epsin might also play a later part, through its binding to Eps15 — a protein that has been detected at the rim of budding coated vesicles¹³.

Understanding all the molecular events that govern the making of a vesicle will require the development of ever more sophisticated approaches, using *in vitro* systems combined with morphological analysis at the ultrastructural level, as exemplified by Ford and colleagues' work¹. This will be complemented by the use of biophysical techniques, some based on high-resolution

imaging, that allow us to study the process in atomic detail¹⁴.

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Virology

Ins and outs

Viruses have a nasty habit of usurping the normal biological processes of their hosts. Take, for instance, adenovirus, which causes mild respiratory infections in humans. This virus takes advantage of a protein that is expressed naturally by the sheets of epithelial cells lining our lungs. It uses this protein, CAR, to latch onto the cells before entering them and multiplying.

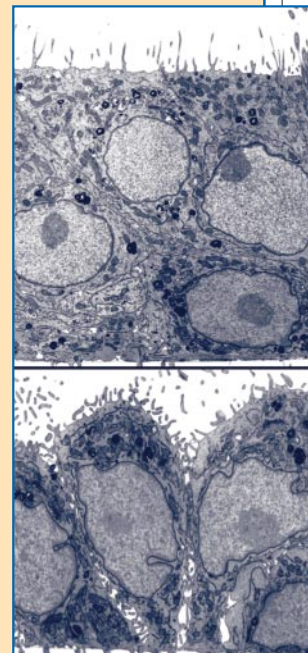
Contrary to expectations, the CAR protein is found, not on the exposed surfaces of the cells, but rather on their sides, where one cell abuts on another. It seems that temporary breaks in the epithelium allow a few viruses to detect CAR and enter cells. But Michael J. Welsh and colleagues suspected that adenovirus's finely honed ability to bind this protein must also serve another purpose. As they describe in *Cell* (**110**, 789–799; 2002), they've now discovered what that purpose is.

Welsh and colleagues used a previously developed *in vitro* model of lung epithelial sheets that is similar to the human airway. They infected these sheets with adenovirus, and found that progeny viruses were released at the sides and base (the basolateral surface)

of a cell, appearing only later on the exposed (apical) surface. The apical surface remained undamaged, suggesting that the viruses travelled up between the cells to escape. Supporting this idea, electron microscopy showed that there were unusual spaces between the cells.

The 'proper' function of the CAR protein is to allow epithelial cells to stick to one another and form a continuous sheet. It was known that the adenovirus fibre protein binds to CAR, allowing the virus to enter cells. Welsh and colleagues wondered whether this fibre protein could also cause the breaks in epithelial sheets that are seen after the production of progeny virus. When adenovirus multiplies in epithelial cells, it produces far more fibre protein than can be incorporated into new viral particles. The authors found that when they added fibre protein to the epithelial sheets, it disrupted cell-to-cell contacts, as seen in these before (top) and after (bottom) pictures.

So they propose the following sequence of events. Adenovirus enters cells through temporary natural breaks in the epithelial sheet, which allow it



to use its fibre protein to bind to CAR. It multiplies in the cells, and progeny are released to the basolateral surface, along with surplus fibre protein. That protein again binds to CAR and prevents it from interacting with other cells in the sheet, thereby making large holes through which the virus can escape into the lungs. Neatly, then, the virus uses the same tools for both entry and exit.

Amanda Tromans

Planetary science

Tracking the martian climate

Alan D. Howard

Like Earth, Mars has experienced long-term fluctuations in climatic conditions. The cause of certain fluctuations is now identified as variation in the planet's astronomical behaviour.

In 1971, the Mariner 9 orbiter revealed extensive layered deposits on Mars at each pole at latitudes above 75°. Later spacecraft provided more detailed views of these deposits, which occur in both the northern and southern hemispheres as broad domes up to 2.5 km thick (Fig. 1). Layers in Earth's ice caps have revealed a detailed record of the climatic history of the past several hundred thousand years, and the martian polar layering presumably carries similar information. This record might tell us about temporal changes in the abundance of water on Mars, and its distribution as liquid, ice and vapour. The inferred climate changes might also provide clues to the age of young features in more temperate martian latitudes, such as gullies on slopes¹ and patterned ground, that may have been formed by water or ice. On page 375 of this issue², Laskar *et al.* provide the first evidence of a specific correlation of the martian sequences with variations in climate driven by changes in the axis and the orbit of Mars.

At the martian north pole, the layered deposits are covered with a perennial cap of water ice with dust mixed in³. At the south pole the perennial cap is much smaller than the layered deposits, and is composed of carbon dioxide ice probably underlain by water ice. A roughly spiral set of troughs and scarps incised into the underlying deposits at the north pole (Fig. 1) define a succession of nearly horizontal layers on the trough slopes facing the equator. The layers have been deposited by accumulation of ice and atmospheric dust in uncertain proportions, possibly incorporating CO₂ in a caged form known as clathrates. The trough slopes have presumably been exposed by ablation of water ice within the layers by evaporation and removal of the dust by wind³.

Explanations for the polar layering have centred on the effects of insolation — the solar radiation received at the martian surface — in regulating the climate. Climate change, in turn, is expected to modulate both the rate at which layers accumulate and their composition, and possibly their removal or thinning by ablation and wind erosion³. Both Earth and Mars are subject to quasi-periodic variations in the characteristics of their spin axes (precession and obliquity) and their orbital eccentricity, due to gravitational interactions with other planets and the Sun⁴. On Earth the variations are modest, with obliquity varying through about 2.5°

and eccentricity from 0.01 to 0.05, but they have been correlated with the major climate changes accompanying the terrestrial ice ages (the Milankovitch theory⁵). Because Mars lacks a large moon and is closer to the massive outer planets, it undergoes much more dramatic orbital variations: during the past 10 million years, obliquity has ranged from about 13° to as much as 47°, and eccentricity from 0.00 to 0.13. These variations occur in a complicated 'beat' pattern involving several frequencies (see Fig. 2 of Laskar and colleagues' paper on page 376).

It has been difficult to decipher the presumed climatic record contained in the martian layered deposits. One issue is the uncertain connection between the orbital variations and processes that could result in the accumulation and erosion of layers. The orbital effects on seasonal and latitudinal

variations in insolation are well understood (see Fig. 2 of Laskar *et al.*). But we know only within broad limits how insolation is linked to conditions such as atmospheric pressure and circulation patterns; dust-storm intensity; cycling of water between the layered deposits, ice in loose sediment and dust on the surface, and groundwater; and rates of accumulation or ablation of ice from polar deposits. Which of the several component cycles of climate variation correlate with the layers has therefore remained unclear.

Imprecise knowledge of the thickness and sequence of exposed layers has also limited their interpretation. Images returned by Viking allowed resolution of the layers only down to about 10–20 m thickness. The increased resolution of the camera on Mars Global Surveyor has permitted measurements to a precision of a few tens of centimetres. But it was not until data were returned from the laser altimeter on this spacecraft that the steepness of the slopes exposing the layers could be reliably estimated, allowing more accurate calculations of layer thickness.

Laskar *et al.*² have statistically matched observed vertical variations in layer brightness in a north polar scarp to calculated temporal variations in polar insolation. The

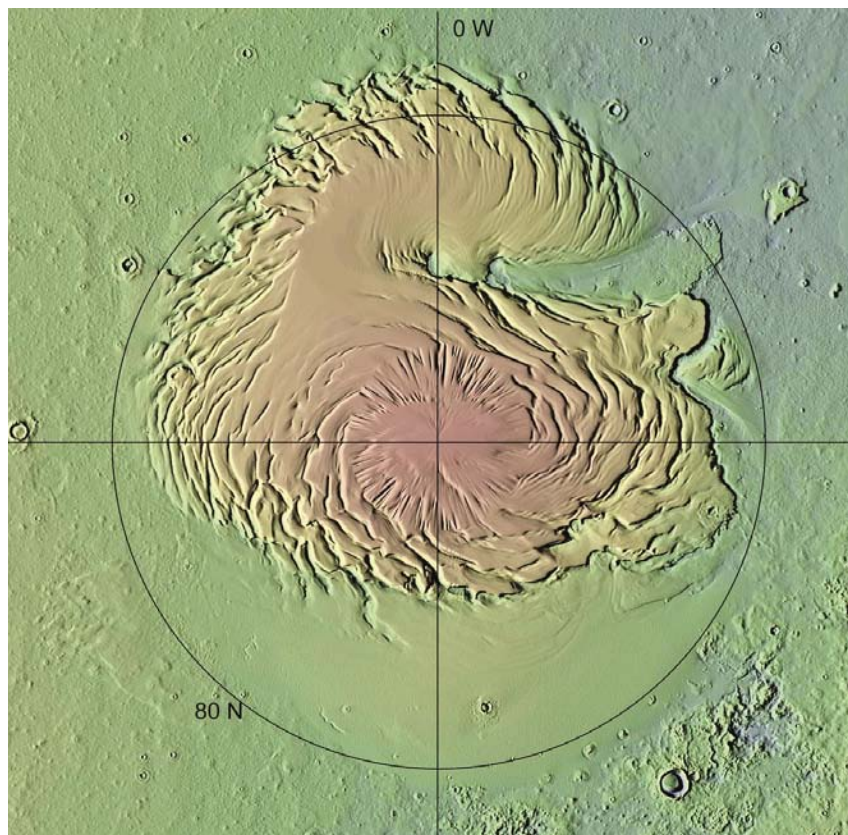


Figure 1 Relief image of the north polar cap of Mars. The cap is underlain by layers of ice and dust exposed in the spiral pattern of troughs, which Laskar *et al.*² relate to astronomically driven climate change. The image is based upon topographic data returned by the laser altimeter on the Mars Global Surveyor spacecraft. The top of the layered deposits is about 2.5 km above the surrounding plains. The radial grooves north of 88° are an artefact of sparse data.

authors use techniques similar to those used in terrestrial correlations between quasi-periodic astronomical effects and climate 'proxies' such as tree-ring thickness, isotope concentrations, and ice and sediment thickness. They find an intriguing match between variation in insolation and layer brightness when the top 350 m of layers exposed in a polar scarp is assumed to correspond to about 0.9 million years of accumulation, giving an average rate of accumulation of about 0.05 cm yr⁻¹.

This estimated rate of accumulation is near the high end of previous estimates (which were based on calculations of the age of surface polar deposits from impact crater density, and on the rates of exchange of atmospheric dust and water vapour between the polar cap and the atmosphere). At 0.05 cm yr⁻¹, the entire 2.5 km of layered deposits could have accumulated within the past 5 million years, astonishingly recently given the 4.5-billion-year age of the planet.

As Laskar *et al.* indicate, the figure of 5 million years could be consistent with the strong seasonal insolation associated with high obliquity that occurred before then: strong insolation could have discouraged layer deposition and ablated any earlier deposits. Possibly related to this, the north polar deposits are underlain by a sand-rich layer that was probably a dune field emplaced at some time when the polar cap was absent⁶. On the other hand, the layered deposits might be much older if deposition of new ice and dust between troughs roughly balanced rates of ablation on the equator-facing scarps³.

The proposed youth of the north polar

layers raises other questions. The density of craters on the deposits at the south pole suggests a surface age of about 100 million years⁷. Why would the north polar deposits have been ablated during high-obliquity cycles while those at the south pole persisted? Where would the water in the northern deposits have gone if the cap has been episodically absent in recent geological time? If distributed globally, the amount of water released might have been enough to cover the planet to a depth of several metres, and might have contributed to the formation of young gullies and ice-related landforms in temperate and equatorial latitudes¹.

Laskar *et al.*² studied layering on one polar scarp. A rich record exists on many other scarps in both the north and south. Further studies must exploit these other sites to examine the consistency of depositional history among the scarps and provide a longer total record of climatic change. In the future, missions that land on the planet and drill into the layered deposits may provide definitive interpretations of the climate cycles on Mars. ■

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Cell biology

Unchaining the condemned

Keith D. Wilkinson

Like prisoners, condemned intracellular proteins are shackled with chains to await their fate. These chains are a signal that the proteins must be destroyed and are removed on protein degradation. New work shows how.

To carry out their functions properly, the proteins in our cells must be in the right place at the right time, and at the right concentration. So it's vital that cells achieve the correct balance between protein synthesis and destruction. Although we understand much about how proteins are made, it is only in the past ten years that we have come to appreciate the complexity of their degradation. Like everything else, proteins outlive their usefulness and, whether damaged or just no longer needed, they are often condemned to destruction by the covalent attachment of another protein, called ubiquitin. When this process fails, it has profound consequences for

events such as cell division, gene expression and the development of cancer¹. Writing on page 403 of this issue and recently in *Science*, Yao and Cohen² and Verma and colleagues³ propose that the ubiquitin must be removed for proteins to be rapidly degraded. The enzyme behind this ubiquitin removal might represent another step in protein degradation that is a good drug target.

Ubiquitin is a 76-amino-acid protein that is present in all eukaryotes (loosely, those species, including humans, whose cells have a nucleus). The attachment of a single ubiquitin molecule to an intracellular target protein is often used as a sorting signal,

or simply to modify the protein's properties. This 'monoubiquitination' can be reversed by certain cellular deubiquitinating enzymes⁴. By contrast, the degradation of many — perhaps even most — intracellular proteins is triggered by the addition of several more ubiquitins to the first. The resulting 'polyubiquitin' chain can be recognized by a binding site (or sites) on the cellular executioner — the proteasome.

The proteasome (Fig. 1, overleaf) is a complex made up of many different proteins; it degrades ubiquitinated proteins and shortens polyubiquitin chains to recycle the ubiquitin. It consists of a large cylindrical particle, with protein-degrading sites in its lumen, and two regulatory particles that cap each end of the cylinder. The regulatory particle binds polyubiquitinated proteins, unfolds them, and feeds them into the lumen of the cylinder, where they are degraded by hydrolysis⁵.

Because the protein to be degraded must enter the proteasome's cylinder through a narrow opening in the end, it is likely that the polyubiquitin chain must be removed to allow the whole protein to be fed through the opening and hydrolysed. Moreover, at least in yeast cells, a lack of free ubiquitin is lethal⁶, so this may be another reason why it must be recycled from substrate proteins.

At least two deubiquitinating enzymes that catalyse the disassembly of polyubiquitin chains are known to be tightly associated with the mammalian proteasome. Neither of these, however, is known to be required for degradation. UCH37 is a member of the ubiquitin carboxy-terminal hydrolase family of enzymes, and cleaves the polyubiquitin chain from its free end, furthest from the attached protein. It has been speculated that this enzyme acts as a molecular clock that slowly shortens ubiquitin chains, allowing the attached protein to be released from the proteasome if there is a delay in efficient degradation⁷. Thus, clogged proteasomes would be cleared and become available for further use. A second deubiquitinating enzyme, USP14, is a member of the ubiquitin-specific protease family. However, little is known about its role or substrates.

Both of these deubiquitinating enzymes contain a thiol (sulphydryl) group in their active sites, and are inhibited by ubiquitin aldehyde — a substrate analogue that reacts with the thiol group. But nearly ten years ago Hershko and his colleagues⁸ described another deubiquitinating activity associated with the proteasome that is insensitive to ubiquitin aldehyde. Yao and Cohen² and Verma *et al.*³ investigated this activity further. Both groups suggest that the enzyme responsible is a metalloprotease — a metal-ion-dependent protein-cleaving enzyme — and that it links deubiquitination to degradation.

The authors found that an aldehyde-insensitive deubiquitinating activity is tightly

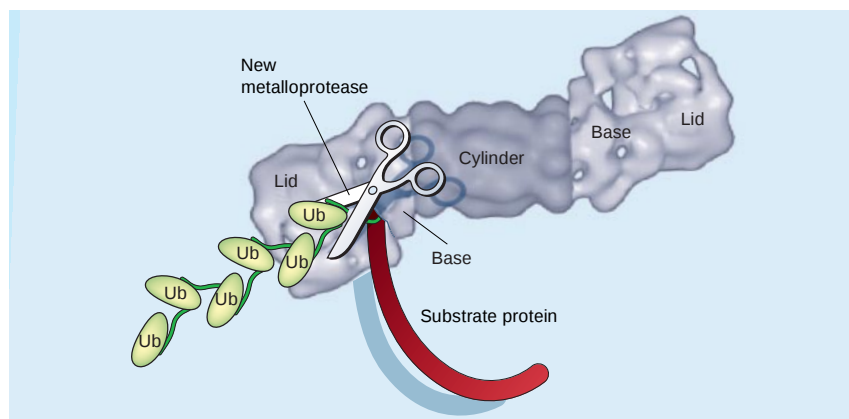


Figure 1 The proteasome — the cellular protein executioner. The proteasome consists of two multiprotein components: the regulatory particle and the cylinder. The regulatory particle itself consists of a base and a lid. Proteins to be degraded are labelled with chains of ubiquitin (Ub); these chains are recognized by the proteasome's lid. The base contains six enzymes that are thought to unfold the substrate protein and move it into the lumen of the cylinder to be degraded. Yao and Cohen² and Verma *et al.*³ find that efficient translocation of the protein into the lumen probably requires removal of the polyubiquitin chain by the proposed metalloprotease activity of the Rpn11 subunit, found within the lid. Figure adapted from ref. 5.

associated with purified proteasomes and efficiently removes ubiquitin from model substrates. This activity depends on the main cellular energy store, ATP molecules, and the results imply that ATP-dependent movement of the substrate into the lumen of the proteasome's cylinder may be necessary to properly position the ubiquitin for cleavage.

Yao and Cohen² also found that, in the absence of the deubiquitination activity, the ubiquitin attached to the substrate was degraded along with the substrate, albeit more slowly. So deubiquitination can become the rate-limiting step in degradation. Using a proteasome inhibitor, Verma *et al.*³ showed that the deubiquitination activity was required to remove ubiquitin from a substrate that (because the proteasome was inhibited) presumably then became trapped in the lumen of the proteasome. Both studies point to the idea that the movement of substrates into the lumen is coupled with deubiquitination.

The two groups also propose that the best candidate for this aldehyde-insensitive enzyme is the Rpn11 protein, also known as POH1, found in the 'lid' of the regulatory particle. Rpn11's active site has features that suggest it is a metalloprotease. The protein bears little resemblance to other deubiquitinating enzymes but is strikingly similar to a protein found in another multiprotein complex, called the COP9 signalosome, which efficiently removes the ubiquitin-like protein Nedd8 from its conjugate with the cullin protein⁹. So Rpn11 may define a new family of deubiquitination-type enzymes.

The finding that removal of the polyubiquitin chain is necessary to fully degrade a condemned protein and to recycle single ubiquitins may explain why both groups^{2,3} observed that mutations of the Rpn11 pro-

tein kill yeast cells. However, it remains to be demonstrated that the putative metalloprotease has enzymatic activity *in vitro*. Another question is what role USP14 plays in the metabolism of proteasome-bound ubiquitin chains. Nonetheless, it is now clear that the unchaining of a condemned protein by deubiquitinating enzymes is a vital part

of degradation, and is a potentially exciting new target for the development of drugs that inhibit proteasome function. Such drugs might seem inherently dangerous — after all, protein degradation is an essential cellular event. But in fact some proteasome inhibitors are already in clinical trials and show surprising efficacy in treating multiple myeloma and other cancers of blood cells¹⁰. At the doses used, these drugs block about 80% of proteasome activity in blood cells, but do not seem to reach other tissues, where they might be toxic.

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Chemistry

Material marriage in electronics

E. W. Meijer and Albert P. H. J. Schenning

Self-organizing molecules can form structures with useful electronic properties. These supramolecular materials combine the benefits of polymers with those of organic crystalline systems.

To create new electronic devices, physicists and engineers have had to rely increasingly on the originality of chemists in designing, synthesizing and characterizing molecular systems possessing useful properties. Exciting results have been obtained with both polymer-based light-emitting diodes and organic transistors, in which molecular-scale layers are deposited from the vapour phase. But these two semiconducting classes of material have different virtues. The particular advantage of the polymer systems is that they are cheap and highly 'processable' — that is, easy to synthesize, manipulate and incorporate into devices. The advantage of the organic materials is the precise ordering of their thin crystalline layers, which supports the high charge-carrier (electron and hole) mobility essential to electronic performance.

The dream for many scientists is to bring these two features together in one class of

materials, and to produce easy-to-process yet highly ordered molecular systems. On page 384 of this issue¹, Percec *et al.* describe a novel and clever way of doing this. As with most such research, 'π-conjugated systems' are the key theme. Put simply, these are materials containing electrons that are not tied up in individual covalent bonds, so that they can travel freely over the molecular framework and hop from molecule to molecule. Percec *et al.* have used a remarkably simple strategy to 'program' various electronically active molecules into liquid-crystalline columns containing cores with 'π-stacks' that display high electron and hole mobility. By this they have taken an important step towards the creation of self-organizing systems with favourable carrier mobility.

It is 25 years since Chandrasekhar and co-workers published their seminal paper² describing the properties of the first 'discotic' liquid crystals. These disc-like molecules

self-organize into stacks or columns, which typically order themselves into hexagonal lattices. It was 1994 before the first example³ of fast light-induced conductivity in such systems was obtained. But since then the ability of synthetic chemists to bring molecules together selectively, in well-defined architectures, has increased tremendously.

The upshot has been that materials scientists have discovered the world of supramolecular chemistry, which has the potential to marry the best characteristics of high-molecular-weight polymers and low-molecular-weight organic molecules. As their name implies, supramolecular materials self-assemble and are therefore often easier to synthesize and process than crystalline materials. And provided the individual building-blocks are carefully 'programmed', the self-assembly should yield structures and molecular orientations that are suitable for the desired function. For example, the macroscopic orientation of the π -conjugated components in organic devices is of central importance for determining the direction of charge-carrier mobility, as has been demonstrated for semiconducting polythiophenes⁴.

Combining the strengths of classical polymers and single crystals will require new thinking. The way forward is to take functional, disordered molecules and allow them to self-assemble — through information programmed into the molecule — into well-defined arrays. To obtain a general scheme for making such self-processable organic materials, Percec *et al.*¹ apply several different concepts from supramolecular chemistry. The details of the chemistry and the liquid-crystal assembly are shown in Figs 1 and 2, respectively, on page 385. In short, the authors have synthesized fluorine-carrying clusters of highly branched polymers called dendritic wedges. When electron donor and acceptor groups are attached, the wedges self-assemble into supramolecular columns. It is self-assembly of these wedges that drives the liquid-crystal formation of the organic component. Both low-molecular-weight organic materials and polymerized species can be used as donor and acceptor groups. By selecting the right ingredients, the molecules co-assemble into columns in which a donor-acceptor complex is formed in the core of the liquid-crystal column. The fluorinated periphery of the molecules shields the core of the column from external influences such as moisture. Remarkably, even disordered polymers self-assemble into well-defined columns.

Percec and colleagues' in-depth analysis of the liquid-crystalline phases, including the use of X-ray and NMR spectroscopy, provides a wealth of information about the organization of the molecules within the column, as well as showing that the columns are oriented perpendicular to the surface.

These studies reveal that the regularly packed nanocolumns are of high density, with roughly 10^{12} columns per square centimetre. The charge-carrier mobilities, as determined by the time-of-flight method, fall in the range of 10^{-4} – 10^{-3} cm² V⁻¹ s⁻¹, well within the values needed for molecule-based devices. The data showing at high resolution how the system is packed are particularly striking. Using an especially sophisticated NMR technique⁵, a group of the authors in Mainz resolved the distances between different building-blocks in the liquid-crystalline phase to the proton-proton level.

The true significance of the systems described by Percec *et al.*¹ will only become clear when they are incorporated into electronic devices (for transistor applications, the column orientation will preferably be parallel, rather than perpendicular, to the surface). An even more exciting prospect would be the independent use of individual columns, with the goal of producing supramolecular electronics as an alternative to molecular electronics. Several groups, including our own, are currently engaged in such work. Columns with diameters as small as 3–5 nm and lengths of 50–100 nm should self-assemble between electrodes with a single-crystal-like packing, providing alternatives for single-walled carbon nanotubes or inorganic wires. All in all, the concepts outlined by Percec *et al.* constitute a good starting point in the search for supramolecular electronic materials.

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corrections

In Giles Cory and Anne Ridley's article "Cell motility: Braking WAVES" (*Nature* **418**, 732–733; 2002), the wrong attribution appeared for the time-lapse film of a neutrophil chasing a bacterium, provided as a spectacular example of cell motility. The movie was made by the late David Rogers at Vanderbilt University, Nashville, Tennessee (see <http://expmed.bwh.harvard.edu/projects/motility.html>).

In "Global change: Oceanic action at a distance" by Raja S. Ganeshram (*Nature* **419**, 123–125; 2002), the author list for one of the two main papers under discussion (ref. 3) contained an error. The correct citation for that reference is Matsumoto, K., Sarmiento, J. L. & Brzezinski, M. A. *Glob. Biogeochem. Cycles* **16**, 10.1029/2001GB001442 (2002).



100 YEARS AGO

Only one subject — Latin — is really educational in our schools. I do not mean that the average boy reads any Latin author after he leaves school, or knows any Latin at all ten years after he leaves school. I do not mean that his Latin helps him even slightly in learning any modern language, for he is always found to be ludicrously ignorant of French or German... But I do mean that as the ordinary public-school master is really able to give a boy easy mental exercises through the study of Latin, this subject is in quite a different position from that of the others. If any proof of this statement is wanted, it will be found in the published utterances of all sorts of men... who, confessedly ignorant of "the tongues", get into a state of rapture over their school experiences and the efficiency of Latin as a means of education. All this comes from the fact, which schoolboys are sharp enough to observe, that English schoolmasters can teach Latin well, and they do not take much interest in teaching anything else.

From *Nature* 25 September 1902.

50 YEARS AGO

"My particular mission has been to make men of science conscious of their power and influence in shaping civilized life." So wrote Sir Richard Gregory in his message of farewell published in *Nature* of January 7, 1939, when he resigned from the editorial chair which he had occupied with such distinction for so many years. Throughout his long life, which came to a close on September 15, his interest in science for its own sake, and equally for the appreciation by others of its value and significance for humanity, was a driving force which brooked no obstacle... He was a true working editor, marking manuscripts for the printer and seeing the journal through the press week by week with all the attention to detail such work demands. There was never an editorial committee; neither was there even a special panel of reviewers. The entire scientific world, both at home and abroad, has always been *Nature's* willing advisers and critics. Thus, through this valuable freedom from committee control... was Sir Richard able to contribute very largely towards making the journal what it is... He accepted the saying that 'a good editor wears out the soles of his shoes before the seat of his pants'.

From *Nature* 27 September 1952.

Extrasolar planets

Jack J. Lissauer

Natural philosophers have speculated on the existence of worlds around other suns for millennia. Now that real data are available, we find a diversity far beyond that expected by scientists, or science-fiction writers.

NASA/ESA

In the 1960s, with great fanfare, the discovery of first one, and then two Jupiter-like planets in orbit around Barnard's star was announced (Fig. 1). Only 6 light years away — but still too faint to see with the unaided eye — Barnard's star is one of the Sun's nearest neighbours (only the Alpha Centauri system is closer). But by the 1970s, the evidence for these purported planets was discredited. More claims of the discovery of the first extrasolar planet, or 'exoplanet', continued to capture newspaper headlines, but these too failed to stand up to scrutiny.

It was only after decades of false leads that, in 1991, two bona fide extrasolar planets were detected¹, and this discovery has stood the test of time. Exoplanets are small, very faint objects, located close to much brighter stars. The planets themselves have not been seen, but instead they have been identified by the gravitational tugs that they exert on their stars.

About 100 exoplanets are now known; most are comparable in mass to Jupiter, and have orbital periods of a few years or less. Astronomers are amassing a variety of detection techniques to better assess the diversity of planetary systems within our Galaxy. And the hunt is on for a true

analogue of our Solar System that has an Earth-like planet, perhaps harbouring life as we know it.

What is a planet?

Five planets, or 'wandering stars', were known to the ancients: Mercury, Venus, Mars, Jupiter and Saturn. The astronomical revolution brought about by Copernicus, Kepler and Newton showed that these objects were more akin to the Earth than to the Sun and other stars. Thus our home orb was added to the list of known planets. Then, in 1781, the scientific world was taken by surprise when amateur telescope-maker William Herschel announced the discovery of a more distant planet, subsequently named Uranus. In 1800, the small planetary object Ceres was discovered in orbit between Mars and Jupiter, and tens of thousands of even smaller minor planets — asteroids — have since been detected in that region. The planet Neptune signalled its presence through its gravitational effect on the orbit of Uranus, and was first actually seen in 1846. And Pluto, the furthestmost Solar System planet known to us, appeared in a careful optical search carried out by Clyde Tombaugh in 1930.

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NEWS and VIEWS

Barnard's Star

BARNARD'S STAR is the second nearest known stellar system, with apparent visual magnitude 9.5, a parallax of $0''.55$ and a proper motion of $10''.3$ per annum. It has been investigated at the Sproul Observatory of Swarthmore College by P. van de Kamp (*Astronomical J.*, 68, 515; 1963) on photographs taken over a period of forty-five years; 2,413 plates with 8,260 exposures obtained on 609 nights by many observers. Barnard's Star has the largest known secular perspective acceleration in proper motion. This was determined ($-0''.00014$ in R.A., $+0''.00118$ in declination). The residuals of the observations from the predicted positions allowing for proper motion, secular acceleration and parallax were investigated. The residuals showed a periodicity of about 24 years, and indicate the presence of a companion. A semi-axis major of $0''.0245$ and an eccentricity of 0.6 for the main star fit the observations. If the mass of Barnard's Star is 0.15 solar mass, the companion has an orbit with semi-axis major of $2''.4$, or 4.4 astronomical units, and from the displacements of the main star it can be shown that the mass of the companion is 0.0015 solar masses, or 1.6 times the mass of Jupiter. Its apparent visual magnitude would be perhaps 30, far below the reach of existing telescopes.

Figure 1 The 1963 'discovery' of a planet in orbit around Barnard's star is reported in *Nature*. The image above shows the Cone nebula, a star-forming region 2,500 light years from Earth, whose clouds of gas and dust could one day form planets.

The search for more planetary companions to the Sun continues, using direct imaging as well as the indirect signature of gravitational perturbations of the motions of

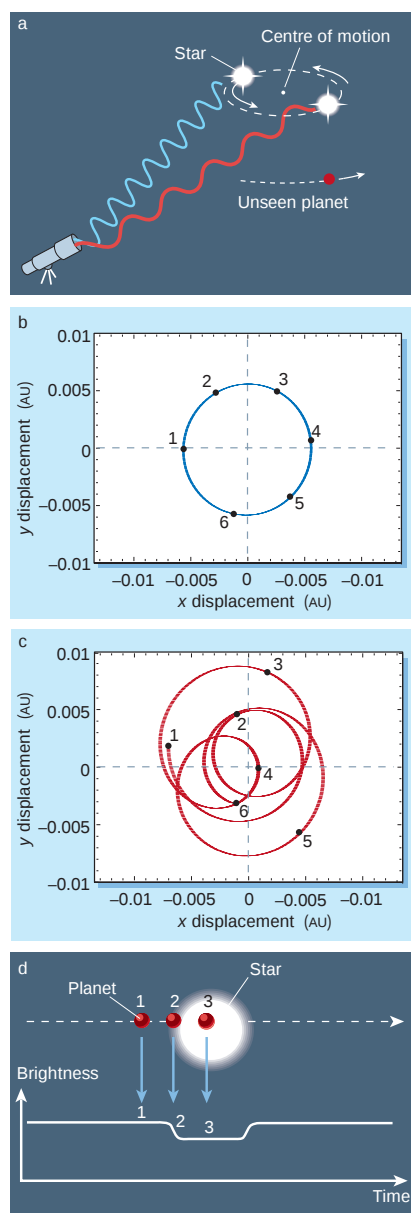


Figure 2 Signatures of exoplanets. a, The Doppler effect. An unseen planet influences the motion of its star: as the star moves towards the observer, the observed frequency of the starlight increases, becoming 'blueshifted'; as the star moves away, the light frequency decreases — becomes 'redshifted'. By detecting these frequency changes, astronomers can infer the presence of the orbiting planet. b, Astrometric data show the effect of Jupiter on the position of the Sun, and c, the combined effect of all nine Solar System planets. Points 1–6 indicate the position in years 2000–2050; 1 astronomical unit (au) is the mean distance between the Earth and the Sun. (Courtesy E. V. Quintana.) d, Partial eclipses of a star by an orbiting planet — or 'transits' — can be picked up through the distinctive dip that is periodically observed in the star's brightness.

known planets, comets and even spacecraft.

Pluto was originally thought to be more massive than the Earth, but subsequent observations showed that it is less than 5% of the mass of Mercury, the smallest of the planets known before 1800 and itself less than 6% of the mass of the Earth. This realization, together with the discovery of many minor planets beyond Neptune during the past decade (the largest of which may be bigger than Ceres), has led astronomers to question exactly how a planet should be defined.

For exoplanets, the question is not whether an object is too small to call a planet (small objects are difficult to detect), but rather whether it is too large. A star maintains itself against gravitational collapse using energy released by nuclear fusion in its interior; only objects at least 7–8% as massive as our Sun can maintain sufficiently high temperatures in their interiors to become stars. In comparison, the most massive planet in our Solar System, Jupiter, has less than 0.1% of the mass of the Sun. Various definitions of a planet have been proposed, some based on mass, or the origins of the body, or on its current orbit. The provisional definition adopted by the International Astronomical Union's working group on extrasolar planets is an object that is in orbit about a star and that is smaller than the limit for deuterium fusion to occur (about 13 times the mass of Jupiter).

How to find a planet

There are several ways to search for exoplanets, and, as planets located many light years away are extremely faint, most methods are indirect — a planet is detected through its influence on the star that it orbits. Different methods are sensitive to different classes of planets and provide complementary information about the planets they find, so most or all of them will contribute to our understanding of the diversity of planetary-system characteristics.

In 1991, Alexander Wolszczan and Dale Frail announced the presence of two planets in orbit about a pulsar¹, in what became the first exoplanet discovery. Pulsars are magnetized, rotating neutron stars, which emit radio waves that appear as periodic pulses to an observer on Earth. It was variations in the arrival times of these pulses that signalled the planets' presence to Wolszczan and Frail. The pulse period can be determined very precisely (stable pulsars rank among the most accurate clocks known), and the mean time of pulse arrival at the telescope receiver can be measured especially accurately for rapidly rotating, millisecond pulsars, whose frequent pulses provide an abundance of data. Even though the pulses are emitted periodically, the times at which they reach the receiver are not equally spaced if the distance between the pulsar and the telescope varies in a nonlinear fashion. The

Earth's motion around the Sun and its rotation cause such variations, and these can be calculated and removed from the data. If periodic variations are still present in the data, they may indicate the presence of companion planets orbiting the pulsar.

But by far the most successful planet-finding method at present is the radial-velocity technique. The wavelength of light emitted by distant stars becomes lengthened or shortened depending on whether the star is moving away from or towards the observer (Fig. 2a). By fitting this 'Doppler shift' in the wavelengths of a large number of features within a star's spectrum, the velocity at which the star is moving towards or away from the observer can be precisely measured. Astronomers then subtract the motion of the telescope relative to the centre-of-mass of the Solar System and other known motions, leaving the radial motion of the target star that results from the tug of its own planets. Precise radial-velocity measurements require a large number of spectral lines, and so are not possible for the hottest stars, which have far fewer spectral features than do cooler stars like the Sun. Moreover, stellar rotation and intrinsic variability (including starspots) are major sources of noise for radial-velocity measurements.

There is another slight drawback to both pulsar timing and radial-velocity measurements. Although both are sensitive to the period and the eccentricity of the exoplanet's orbit, an important quantity to measure for any newly discovered planet is its mass. But both methods yield only the product of the planet's mass (divided by that of the star, whose mass can usually be estimated accurately from its spectral characteristics) and the sine of the angle between its orbital plane and the plane of the sky — $M \sin i$ — and i usually cannot be determined.

Still, in radial-velocity measurements the leading research groups are now achieving a precision of 3 m s^{-1} on spectrally stable stars (this represents a Doppler shift of 1 part in 10^8). Compare this with our own Solar System: Jupiter causes the Sun's velocity to vary with an amplitude of 12.5 m s^{-1} and a period of 11.86 years; Saturn's effect is the next largest, with an amplitude of 2.7 m s^{-1} and a period of almost 30 years. Thus, with current precision, Jupiter-like planets orbiting Sun-like stars are detectable, although these detections require a long timeline of observations (comparable to the planet's orbital period). Planets smaller than Uranus orbiting very close to stars can also be detected. But finding Earth-like planets in Earth-like orbits is well beyond the capabilities of the radial-velocity technique.

Planets can also be detected from the wobble they induce in the motion of their stars projected onto the plane of the sky (Fig. 2b,c). This astrometric technique is most sensitive to massive planets orbiting stars

that are relatively close to us. But here, because the star's motion is detectable in two dimensions, the planet's actual mass, rather than just the combination $M \sin i$, can be measured. Planets in more distant orbits are ultimately easier to detect using astrometry because the amplitude of the star's motion is larger — but then finding these planets requires a longer timeline of observations because of their greater orbital periods.

The detection methods mentioned so far look for a planet's gravitational pull on its star, and so are sensitive to the planet's mass. In contrast, transit photometry detects the amount of starlight that a planet obscures and gives an estimate of the planet's size. If the Earth lies in or near the orbital plane of an extrasolar planet, then, viewed from Earth, that planet periodically blocks a small fraction of the star's light once each orbit. Measuring a star's brightness very precisely can reveal such transits, easily distinguished from other, random effects, such as star-spots, by their periodicity and the distinctive, square-well shape of the brightness variation (Fig. 2d). Although this technique detects only the small proportion of planets that happen to line up in this way, thousands of stars can be surveyed within the field of view of one telescope, so transit photometry should be fairly efficient.

Reaching down further into the box of astronomical tricks, we find the microlensing method, which is being used to investigate the distribution of faint, stellar and substellar bodies within our Galaxy². Microlensing arises from the general relativistic bending of the light from a distant star by a massive object (the lens) passing between the source and the observer. Lensing causes the source to appear to brighten gradually to a few times its usual intensity over a period of weeks or months. If the lensing star has planetary companions, then these less massive bodies would produce brief enhancements in the brightness, provided that the line of sight from Earth passes close to the planet. Under favourable circumstances, planets as small as Earth could be detected. But we would only be able to make statistical estimates of the properties of individual planets, and often even of the stars that they orbit, because of the many parameters that influence microlensed light³.

So there are many indirect ways to find planets beyond the Solar System, but what about imaging an exoplanet directly? Distant planets are very faint and located near much brighter objects (the star or stars that they orbit), making them extremely difficult to image. The reflected starlight from planets similar in orbit and size to those in our Solar System is roughly only one-billionth as bright as the star, although the contrast decreases a thousandfold for thermal, infrared radiation. Scattering of light by telescope optics and atmospheric variability

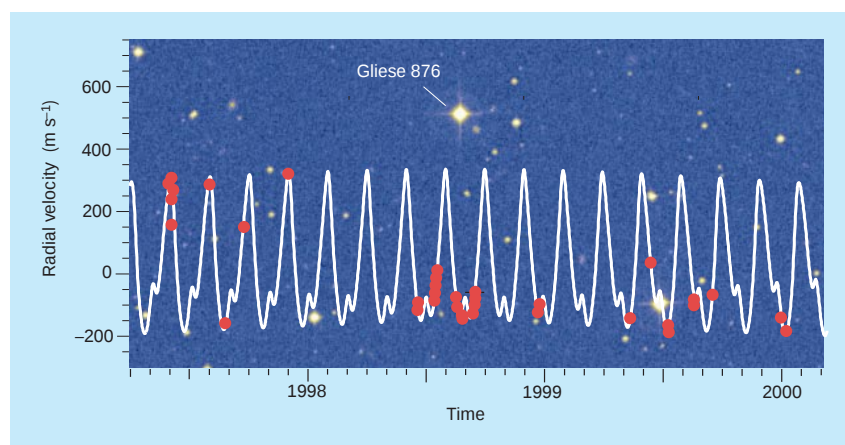


Figure 3 Data from the Keck telescope in Hawaii (red points) show the variation over time of the radial velocity of the star Gliese 876. The white curve is the best fit to the data points, implying that there are two unseen planets perturbing the motion of the star and each other. (Courtesy E. J. Rivera.)

on Earth add to the difficulty. Nonetheless, technological advances such as adaptive optics should eventually permit imaging and spectroscopic studies of planets orbiting nearby and/or younger, brighter stars.

Ground-based searches using all of these techniques are in progress, and for the future, higher-precision astrometry, transit, microlensing and imaging surveys using spacecraft are being considered. There are still other techniques to explore. Precise timing of the eclipses of eclipsing binary stars has the potential to reveal the masses and orbits of unseen companions. Spectroscopy could be used to identify gases that would be stable in planetary atmospheres but not in stars, and Doppler variations of such signals could yield planetary orbital parameters. Radio emissions similar to those detected from Jupiter could reveal the presence of extrasolar planets. And, of course, artificial signals from an alien civilization could betray the presence of the planets on which they lived (and they might even be willing to provide us with substantially more information).

Observed exoplanets

Three small planets — the first and the smallest exoplanets known — orbit the pulsar PSR1257+12, a rapidly rotating neutron star around 1.4 times the mass of the Sun and between 2,000 and 3,000 light years from Earth (placing it in our general region of the Milky Way Galaxy, but not a close neighbour even by interstellar standards). The first two to be found have orbital periods of a few months, small eccentricities, and masses a few times as large as the Earth (here we can estimate the actual masses; the star's response to orbital changes produced by mutual gravitational interactions of the planets provides estimates of the masses independent of the orbital inclination). The minimum mass of the inner planet ($M \sin i$) is only slightly more than that of the

Earth's Moon, and it completes its orbit in just under a month⁴.

So far, all other extrasolar planets for which there is good evidence have been discovered using the radial-velocity (Doppler) technique, although other methods have given tantalizing hints. These exoplanets are much more massive than those around PSR1257+12 and orbit normal, hydrogen-burning stars. Most of these stars have masses within a few tens of per cent of that of our Sun and are situated between 20 and 200 light years away — much closer than the pulsar with known planets, but still not our very closest neighbours. The first such exoplanet was discovered⁵ around the star 51 Pegasi (which is slightly less massive than our Sun and a few billion years older) by Michel Mayor and Didier Queloz in 1995. For this planet, $M \sin i$ is 45% of the mass of Jupiter (M_J) and its orbital period is just 4.23 days — less than a twentieth that of Mercury, the closest planet to our Sun. Several similar planets have been found subsequently, implying that about 1% of Sun-like stars are orbited by Jupiter-like planets with orbital periods of less than one week.

Of the 100 exoplanets now known (with the exception of the pulsar planets), their minimum masses (strictly, $M \sin i$) range from about 40 Earth masses (equivalent to $0.12 M_J$, or a bit over twice the mass of Neptune, the third most massive planet in our Solar System) for the planet orbiting the Sun-like star HD 49674, right up to the maximum allowed by the definition of a planet. Most of the observed exoplanets have masses close to (within a factor of a few of) Jupiter's mass, because larger planets are scarcer and smaller ones are more difficult to detect. The only exoplanet so far discovered with a period exceeding that of Jupiter orbits the star 55 Cancri with a 14-year period and $M \sin i = 4 M_J$.

The actual size has been measured for only one exoplanet. The close companion to

Box 1 The Kepler mission

The detection of Jupiter-like planets generates great scientific and popular interest, but finding planets similar to Earth would be even more exciting. In December 2001, NASA selected the Kepler mission for this task, using transit photometry in space.

Spacecraft are expensive, but there are some observations that just cannot be made from the ground. The effects of the Earth's atmosphere limit photometric precision to roughly 0.1%, which means that although transits by Jupiter-sized planets can be detected from the ground, those by Earth-sized planets cannot¹⁰. Far greater precision is achievable above the atmosphere, as shown by transit observations of HD 209458 by the Hubble Space Telescope.

With its 0.95-m photometer, Kepler will observe the brightness of 100,000 stars almost continuously for four years, with an extraordinarily large field of view covering

105 square degrees (500 times the area of the Moon). Ultra-high-precision photometry will be achieved by spreading the individual stellar images over dozens of pixels on the device's detectors and by correcting for systematic errors.

The Kepler mission has been designed to be capable of detecting Earth-sized planets around enough stars that a null result would prove that systems like our Solar System are rare. If, on the other hand, our Solar System is typical, Kepler should find about 50 Earth-like planets. This number more than doubles if planets 20% larger in radius than the Earth are common, and increases many times over if terrestrial planets commonly orbit stars more closely than Mercury does the Sun. On the basis of statistics obtained from radial-velocity surveys, more than 100 inner-orbit, giant planets should be seen in transit, and most of these



will also be detectable by the variations in light that they reflect from their stars. The masses of many of these inner planets will be determined using radial-velocity measurements.

Kepler's first planet detections — dozens of giant planets in inner orbits — should be made within the first

two months of launch. But detecting the first true Earth analogues will take 3–4 years of observations, because the orbital periods of such planets are longer and more repetitions of the transits must be observed to separate the signal from the noise in the data. Stay tuned for exciting news at the beginning of the next decade!

HD 209458 (another Sun-like star) was first detected using the Doppler technique but was later observed during transit in front of its star. Measurement of the transit shows that this planet has a radius about 1.35 times that of Jupiter. Moreover, as the tilt of its orbit is known from the transit duration, the planet's actual mass ($0.65 M_J$), rather than merely the product $M \sin i$, is known. Together, the mass and size of a planet reveal its density, which implies that the planet orbiting HD 209458 is composed primarily of hydrogen, the lightest and most common element in the Universe, and also the primary constituent of Jupiter and Saturn.

The exoplanetary system of greatest interest to me is that in orbit about Gliese 876. At about one-third the mass of our Sun, this faint red orb is by far the least massive star known to possess any planets. Gliese 876, only 15 light years from Earth, is also the nearest star for which an exoplanet has unambiguously been found. It is one of less than a dozen stars known to have multiple planets⁶, and the only normal star for which the mutual gravitational perturbations of the planets are clearly evident in the data^{7,8} (Fig. 3).

More to come

The pace of planet discoveries using radial velocities will continue to increase for at least the next few years, as more observers obtain the spectrometers and the skills to achieve the high Doppler precision required. Smaller inner planets will be detected as precision improves, and planets with longer periods

will be found as the data sets grow longer. This should include planets that are true analogues of Jupiter.

Transit photometry, which has already been used to observe the planet orbiting HD 209458 both from the ground and from space, should bear fruit in planet detection in the near future. Several groups are conducting transit searches for close-in giant planets from the ground, mostly using wide-field telescopes with diameters smaller than 25 cm. Both NASA and its European counterpart, ESA, are looking into missions to image Earth-like planets before 2020. A few small telescopes that are designed primarily for studying stellar properties will be launched into space during the next five years and should be able to find planets only a few times the radius of Earth. In 2007, NASA will launch the Kepler mission, which will be capable of detecting true Earth analogues (Box 1). The precision of astrometry from the ground is improving as large interferometers are being built, and even higher-precision astrometry should be achieved from special-purpose spacecraft, but as astrometry is more sensitive to planets far from stars, observations will need to be conducted for years to observe a full planetary orbit.

With technological advances in the coming years, we shall learn more about how common planets are, and their distributions of size, mass and orbital properties, as well as their densities, colours and atmospheric compositions. We may find that our Solar System, and our own planet, are not that special. But it would be piling speculation on specula-

tion to foresee the discovery of life elsewhere.

Although broad classes of future discoveries can be confidently predicted, the particulars cannot. This is because the most successful planet-formation theories are designed to explain the observed properties of planetary systems — the uncertainties in initial conditions, and the complexity of the physics and chemistry of star and planet formation, preclude detailed modelling from first principles⁹. Extrapolation of observed distributions is highly unreliable if the processes creating them are not fully understood. The theorists need more data, and after decades of trying, the observers are now providing a bountiful harvest. ■

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Related sites

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- ♦ exoplanets.org
- ♦ www.kepler.arc.nasa.gov
- ♦ obswww.unige.ch/~udry/planet/planet.html
- ♦ www.astro.psu.edu/users/pspm/arecibo/planets/planets.html

Visual structure of a Japanese Zen garden

The mysterious appeal of a simple and ancient composition of rocks is unveiled.

The dry landscape garden at Ryoanji Temple in Kyoto, Japan, a UNESCO world heritage site, intrigues hundreds of thousands of visitors every year with its abstract, sparse and seemingly random composition of rocks and moss on an otherwise empty rectangle of raked gravel¹. Here we apply a model of shape analysis in early visual processing^{2,3} to show that the 'empty' space of the garden is implicitly structured and critically aligned with the temple's architecture. We propose that this invisible design creates the visual appeal of the garden and was probably intended as an inherent feature of the composition.

Created during the Muromachi era (AD 1333–1573), a period of significant innovation in the visual arts in Japan, the unknown designer left no explanation for the layout of the Ryoanji garden (Fig. 1). The rocks have been considered to be symbolic — representing, for example, a tigress crossing the sea with her cubs, or strokes of the Chinese character meaning 'heart' or 'mind'⁴. Such symbolic interpretations do



Figure 1 The Zen garden at Ryoanji Temple in Kyoto, Japan, showing the simple arrangement of rocks that constitutes its design.

not relate to the experience of visually perceiving the garden, however, and provide little insight into the attraction that it holds even for naive viewers.

To examine the spatial structure of the Ryoanji garden, we computed local axes of symmetry using medial-axis transformation^{2,3}, a shape-representation scheme that is used widely in image processing as well as in studies of biological vision. To understand the concept of medial-axis transformation, imagine drawing the outline of a shape in a field of dry grass and then setting it alight: the medial axis is the set of points

where the inwardly propagating fires meet. It has been shown that humans have an unconscious visual sensitivity to the axial-symmetry skeletons of stimulus shapes⁵.

The result of transforming the garden's composition is shown in Fig. 2, in which the dark lines indicate loci of maximal local symmetry. The overall structure is a simple, dichotomously branched tree that converges on the principal garden-viewing area on the balcony. The connectivity pattern of the tree is self-similar, with the mean branch length decreasing monotonically from the trunk to the tertiary level. Both features are reminiscent of actual trees.

The trunk of the medial axis, along which the view of the garden provides maximal Shannon information about the scene⁶, passes close to the centre of the main hall, which would traditionally have been the preferred point from which to view the garden⁴. We found that imposing a random perturbation of the spatial locations of individual rock clusters in the garden layout destroys these special characteristics of the medial-axis skeleton (see supplementary information), supporting the idea that the origin of the structure of the visual ground was not accidental.

There is a growing realization that scientific analysis can reveal unexpected structural features hidden in controversial abstract paintings^{7,8}. We have uncovered the implicit structure of the Ryoanji garden's visual ground and have shown that it includes an abstract, minimalist depiction of natural scenery. We believe that the unconscious perception of this pattern contributes to the enigmatic appeal of the garden.

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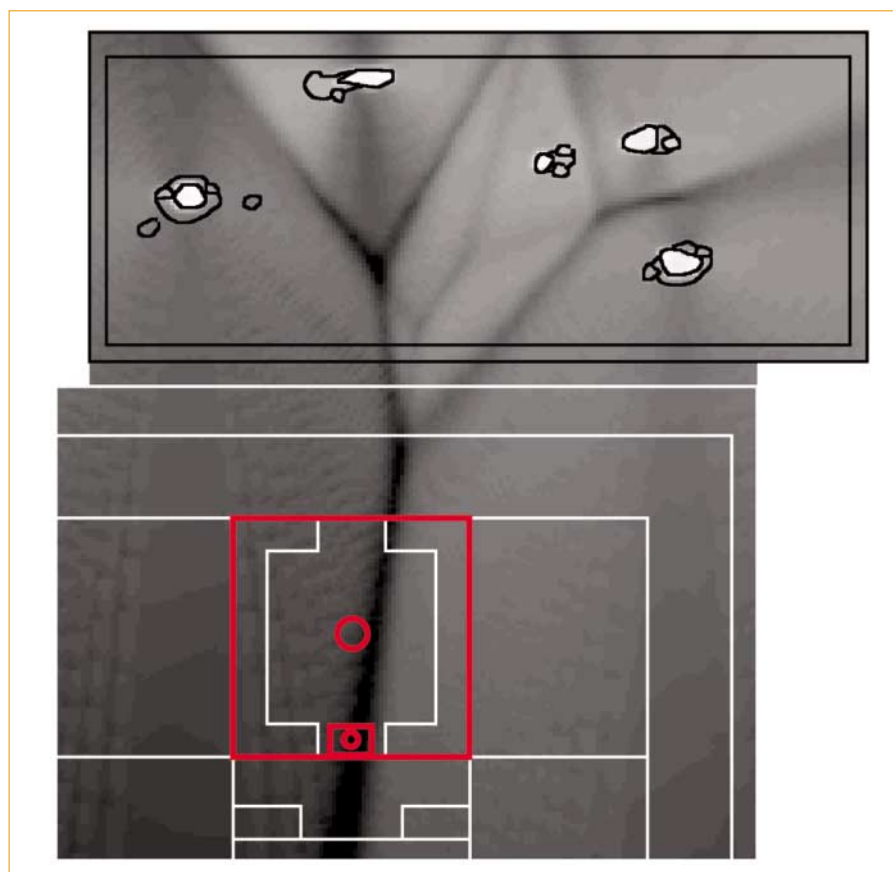


Figure 2 Medial-axis transformation of the layout of the Zen garden, showing the rock clusters (top) and building plan (AD 1681) of the temple (outlined in white). Red square, the main hall; circle, the traditionally preferred viewing point for the garden; rectangle, alcove containing a Buddhist statue. If the positions of the rock clusters are rearranged randomly, features that were incorporated deliberately into the original design of the garden are destroyed (see supplementary information).

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COMMUNICATIONS ARISING

Fruitflies

Pigmentation and mate choice in *Drosophila*

Many species of the fruitfly *Drosophila* are either sexually dimorphic for abdominal pigmentation (the posterior segments in males are black and those of females have thin dark stripes) or sexually monomorphic for this pigmentation (both sexes show striping). Kopp *et al.*¹ report a correlation in two *Drosophila* clades between the expression of the *bric-à-brac* (*bab*) gene, which represses male-specific pigmentation in *D. melanogaster* females, and the presence of sexually dimorphic pigmentation. They suggest that sexual selection acted to produce sexual dichromatism in *Drosophila* by altering the regulation of *bab*, on the grounds that *D. melanogaster* males show a strong mate preference for females with lightly pigmented abdomens, and that this discrimination helps to maintain sexual dichromatism by preventing males from wasting time by courting other (darkly pigmented) males. Here we show that the mate discrimination observed by Kopp *et al.*¹ may in fact have resulted from the nature of the strains and comparisons they used in their study and so could be irrelevant to mate choice in nature.

Kopp *et al.* did not record the specific pairs of female strains used in their 'light versus dark' comparisons (A. Kopp, personal communication), so we could not repeat their experiments exactly. They did, however, use inbred stocks or genetic strains that were not controlled for their genetic background, so that mate choice could be affected by many factors besides pigmentation. We carried out two sets of experiments in which we eliminated this possibility by using females with homogeneous genetic backgrounds derived from the wild. In contrast to Kopp *et al.*¹, we found no evidence that males choose less-pigmented females.

We replicated Kopp and colleagues' methods¹ by placing one wild-type male in a vial containing two virgin females that had different degrees of abdominal pigmentation (all flies were 4 days old), and observing each pair for 30 min. In all vials in which matings occurred, we scored the degree of pigmentation of the A5 and A6 abdominal segments of mated and unmated females using the procedure described by David *et al.*². This method generates pigmentation scores ranging from zero (no pigmentation) to 20 (both segments 100% pigmented).

In our first experiment, we compared two

types of female: those with wild-type *bab* function (normal, light pigmentation) and those with only one functional *bab* copy (*bab*⁻/*bab*⁺ heterozygotes; darker, male-like pigmentation). Chromosomes either containing or lacking the *bab* locus were placed in a wild-type genetic background derived from a *D. melanogaster* stock founded by females collected during 2000 in Arkansas and Louisiana ('ArkLa'). Dark and light females were respectively produced by mating ArkLa males with females from two deficiency strains, Df(3L)Ar12-1 and Df(3L)Ar11. (The former strain was also used by Kopp *et al.*) Both deficiencies are similar in size and were created in the same genetic background, but Df(3L)Ar12-1 deletes the *bab* locus, producing dark heterozygous females (average pigmentation score, 16.4 ± 0.09 (s.e.)), whereas females heterozygous for Df(3L)Ar11, which does not delete the *bab* locus, are lighter (average score, 11.2 ± 0.15).

ArkLa males that were given a choice between *bab*⁻ and *bab*⁺ heterozygous females did not discriminate between these types (94 'dark' matings, 88 'light'; $\chi^2 = 0.2$, $P = 0.67$). These results differ significantly ($G = 38.3$, $P < 1 \times 10^{-9}$) from the combined results of Kopp *et al.*¹, who observed 23 'dark' and 105 'light' matings.

In our second experiment, we produced females of varying pigmentation in the F₂ generation of a cross between an outbred stock of *D. melanogaster* collected in Winters, California, during 2000 and a 'light' female stock produced by combining two inbred lines from the same locality and collected in 2000 (S. Nuzhdin). Males from the outbred stock were given a choice between dark and light F₂ females, with mean pigmentation scores of 11.9 ± 0.17 and 7.5 ± 0.24 , respectively. Again, males showed no significant discrimination between dark and light females (81 'dark' matings, 61 'light'; $\chi^2 = 2.82$, $P = 0.095$).

Our two replicate experiments were statistically homogeneous ($G = 0.94$, $P = 0.33$), but our combined data differed significantly from those of Kopp *et al.* ($G = 52.0$, $P < 1 \times 10^{-10}$). Far from showing a strong preference for light females, our wild-type males showed an insignificant tendency to mate with darker females.

We suggest that Kopp and colleagues' results may be attributed to their comparing mutant or inbred strains with dissimilar genetic backgrounds, so that 'light' and 'dark' females in each trial differed in many of their genes. This idea is supported by the extraordinarily high proportion of trials observed by Kopp *et al.* in which neither

female mated (42 out of 170, 24.7%; A. Kopp, personal communication), compared with the low proportion of such trials in our experiments (14 out of 324, 4.3%). This difference is highly significant ($G = 43.8$, $P < 1 \times 10^{-10}$). Although sexual selection may account for the differences in pigmentation among *Drosophila* species, we find no evidence that it operates in *D. melanogaster* in the way suggested by Kopp *et al.*

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Kopp *et al.* reply — To appreciate how new morphological traits arise in the course of evolution, we need to understand both the genetic basis of phenotypic changes and the selective forces that promote them. We presented evidence that evolutionary changes in the regulation of the *bab* gene could account for the origin of sexually dimorphic abdominal pigmentation in *D. melanogaster*; we also investigated whether sexual selection could explain the origin and maintenance of this trait.

We found that, given a choice between wild-type and *bab*-mutant females (which have ectopic male-like pigmentation), *D. melanogaster* males discriminated in favour of normally pigmented females. This effect was observed in several combinations of *bab*-mutant and wild-type strains, but was abolished when *white*-mutant males, which are effectively blind, were used in mate-choice experiments. On this basis, we suggested that sexual selection against darkly pigmented females can account for the maintenance of sexual dimorphism.

However, Llopert *et al.* argue that this mechanism is unlikely to operate in nature. The difference between our findings is presumably due to the choice of model fly strains. As Llopert *et al.* point out, both the males and females used in our experiments were derived from highly inbred laboratory strains, and extrapolation to natural populations seems not to be supported.

The questions remain — why did male-specific pigmentation evolve in *D. melanogaster* but not in other *Drosophila* lineages? Why is it absent in females? And what selective pressure has maintained this dimorphism for over 20 million years? For now, the answers are that we do not know.

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Curvature of clathrin-coated pits driven by epsin

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Clathrin-mediated endocytosis involves cargo selection and membrane budding into vesicles with the aid of a protein coat. Formation of invaginated pits on the plasma membrane and subsequent budding of vesicles is an energetically demanding process that involves the cooperation of clathrin with many different proteins. Here we investigate the role of the brain-enriched protein epsin 1 in this process. Epsin is targeted to areas of endocytosis by binding the membrane lipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂). We show here that epsin 1 directly modifies membrane curvature on binding to PtdIns(4,5)P₂ in conjunction with clathrin polymerization. We have discovered that formation of an amphipathic α -helix in epsin is coupled to PtdIns(4,5)P₂ binding. Mutation of residues on the hydrophobic region of this helix abolishes the ability to curve membranes. We propose that this helix is inserted into one leaflet of the lipid bilayer, inducing curvature. On lipid monolayers epsin alone is sufficient to facilitate the formation of clathrin-coated invaginations.

Clathrin-mediated endocytosis is essential for many cellular processes, including synaptic vesicle recycling, nutrient uptake and removal of receptors and ion channels from the cell surface^{1–5}. The formation of a new vesicle is thought to be driven by both the polymerization of the coat protein clathrin into a cage-like structure and the selection/modification of membrane lipids^{5,6}. Epsin 1 is implicated in clathrin-mediated endocytosis by binding to the coat components, clathrin, Eps15 and the AP2 complex (Fig. 1a)^{7–9}. There are now at least four known mammalian epsins with different expression patterns (epsins 1–3 and epsin R; see <http://www.ncbi.nlm.nih.gov> or <http://www.kazusa.or.jp/en/>). The epsin 1 homologue in *Drosophila* (*liquid facets*) is an essential gene and as with yeast epsins it has been implicated in endocytosis^{10,11}. The most conserved feature of the epsin family is the epsin N-terminal homology (ENTH) domain. This domain is structurally homologous but distinct from the AP180 N-terminal homology (ANTH) domain found at the N termini of AP180, CALM and HIP. Both domains bind to PtdIns(4,5)P₂ (refs 12, 13).

Tubulation of liposomes

Although both the ANTH domain of AP180 and the ENTH domain of epsin bind to lipids they have distinct effects on liposome shape. Epsin or its ENTH domain converted liposomes made from total brain lipids (Folch extract containing 10% phosphoinositides) into tubules (Fig. 1b). In contrast, the ANTH domain of either AP180 or CALM had no effect on liposome morphology. The outer diameter of the tubules that formed owing to the action of the ENTH domain on liposomes was approximately one-third that formed by the action of either dynamin (not shown) or the N-terminus of amphiphysin (Fig. 1b). Addition of the ENTH domain to synthetic liposomes containing 10% PtdIns(4,5)P₂ (but not liposomes substituted with 10% phosphatidylserine (PtdSer)) also induced tubulation, but the tubules were frequently irregular and many were fragmented into small vesicles (not shown). To begin to understand these effects of epsin we investigated the structural basis of epsin binding to phosphoinositol head groups.

PtdIns(4,5)P₂ binding by epsin

The epsin ENTH domain was crystallized in the presence of inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃) and the structure was solved at 1.7 Å resolution (Fig. 2a; See also Supplementary Information Table 1). Compared with an earlier structure of this domain

solved in the absence of a lipid head group¹⁴ (Fig. 2a, middle structure), a new helix became ordered at the N-terminus, which we have named 'helix zero'. There is also a small rotation in α 8 due to different crystal packing. Helix 0 (α 0, residues 3–15) folds back across the face of α 1 and the loop between α 1 and α 2. This creates a deep basic groove that forms the binding pocket for the Ins(1,4,5)P₃ ligand (Fig. 2a, c). The ligand is coordinated by residues from α 0, α 1, the α 1– α 2 loop, α 3 and α 4, and all three phosphates are multiply coordinated (Fig. 2b). This coordination is consistent with NMR data, although α 0 was undefined in the structure¹². We obtained an additional crystal form in the presence of Ins(1,4,5)P₃ (solved to 1.57 Å and identical to that solved by ref. 14 in the absence of ligand), which contained no density for bound ligand or α 0. These data indicate that binding of Ins(1,4,5)P₃ and α 0 formation are coupled. The increase in α -helicity induced by the inositol head group was confirmed by circular dichroism spectroscopy (data not shown). α 0 is amphipathic, displaying a series of hydrophobic residues (L6, M10, I13) on its outer surface (Fig. 2c; see also Supplementary Information Fig. 1), while the head group is coordinated by its inner surface. This helix is orientated such that it has the potential to interact with the membrane.

The coordination of Ins(1,4,5)P₃ in the crystal structure suggested that epsin is specific for particular head groups, and we confirmed this by affinity measurement using isothermal titration calorimetry (Fig. 3a). We found head-group binding only if the inositol bears a phosphate on both positions 4 and 5, as binding of Ins(1,4)P₂ and Ins(1,5)P₂ was not detected. Ins(1,4,5)P₃ bound with an affinity of $3.6 \pm 0.4 \mu\text{M}$. In the crystal structure residues R8, H73 and the main chain N of N30 coordinate phosphate 4, and K11, R25 and R63 coordinate phosphate 5 (Fig. 2b, c). This coordination of the ligand by residues on the inner surface of helix 0 yields a large binding enthalpy that compensates for the entropic penalty of the formation of helix 0 (Supplementary Information Table 2). Epsin bound InsP₆ with a higher affinity than Ins(1,4,5)P₃ ($0.55 \mu\text{M}$ compared with $3.6 \mu\text{M}$) and with a 1:1 stoichiometry. The short chain lipid di-O-octanoglycerol-phosphatidylinositol-4,5-bisphosphate (diC₈PtdIns(4,5)P₂) also had a higher affinity than its corresponding head group, implying small additional contributions by the glycerol backbone, although the head group is still the main determinant of the strength of the interaction. Head-group binding measured by calorimetry, and lipid binding measured by pelleting with liposomes, showed the same specificities (ref. 12, Fig. 3b and

data not shown). For comparison, the ANTH domain of AP180 had an affinity of $30 \mu\text{M}$ for $\text{Ins}(1,4,5)\text{P}_3$ and $5.1 \mu\text{M}$ for InsP_6 . In contrast to epsin, two ANTH domains bound to each InsP_6 molecule, indicative of a binding site exposed on the surface of the protein (see Fig. 2a), in agreement with our previous observations¹³ and the much smaller entropic loss (Supplementary Information Table 2). For this reason and on the basis of consensus sequences surrounding the AP180 lipid-binding site we propose the subdivision of the epsin/AP180 families. The family of ANTH domain proteins can be defined by the consensus sequence (K/G)A(T/I) x_6 (P/L/V)KxK(H/Y). The mammalian ENTH domain family can also be defined by a consensus over the same region, (D/E)AT x_2 (D/E)PWGP. This takes into account that not all epsins necessarily bind $\text{PtdIns}(4,5)\text{P}_2$, indeed we predict that *Drosophila*

epsin-like protein and its mammalian homologue, which we call epsinR (epsin-related protein, KIAA0171), bind to a less phosphorylated head group (Fig. 2d).

Cell expression studies of epsin 1

In vivo, expression of the epsin ENTH domain in COS cells gave many intensely stained rod-like profiles (not seen with $\text{PtdIns}(4,5)\text{P}_2$ binding mutants), which may be indicative of folding in the plane of the membrane or tubulation (Fig. 4). Full-length epsin showed either a general cytoplasmic distribution or accumulation in puncta on the plasma membrane⁹ (Fig. 4). The puncta co-labelled with antibodies for the AP2 complex (see enlargements), clathrin, Eps15 and dynamin, but not for the AP1 complex, the early endosome marker EEA1, or the Golgi marker GM130 (Supplementary Information Fig. 2 and see <http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/IF>). These puncta probably represent endocytically incompetent coated-pits, because dynamin is not present in mature vesicles and the puncta were found on the plasma membrane (confocal sectioning not shown). An inhibition of coated vesicle formation by epsin was confirmed using the suspension cell line RPMI 1788 cells (Fig. 3d). Cells transfected with epsin or with the AP180 carboxy terminus¹³ had reduced numbers of coated vesicles, whereas vesicles accumulate with the uncoating mutant auxilin N844 (ref. 15).

To probe the function of $\text{PtdIns}(4,5)\text{P}_2$ binding we made a double mutant, R63L H73L, that no longer bound to liposomes containing $\text{PtdIns}(4,5)\text{P}_2$ or to $\text{Ins}(1,4,5)\text{P}_3$ (Fig. 3a, b). The expression pattern of the full-length mutant in COS cells was now always cytoplasmic and the AP2 complex tended to aggregate (Fig. 4, third row) whereas clathrin remained largely unaltered with a punctate distribution (not shown). Transferrin endocytosis was inhibited in more than 90% of these cells, even in cells with a low level of protein expression. Thus targeting of epsin to the plasma membrane by phosphoinositide binding is linked to correct localization of the cargo recruitment complex AP2. The retention of clathrin puncta in cells expressing R63L H73L shows that other proteins can maintain clathrin recruitment.

In contrast to R63L H73L epsin, we reported previously that overexpression of the AP180 C-terminus caused a redistribution of both the perinuclear and plasma membrane pools of clathrin¹³. This correlates with the greater binding capacity of AP180 C-terminus for clathrin whereas epsin binds better to the AP2 complex (Fig. 3c; see also ref. 16). The epsin used in our experiments (epsin 1) had no effect on AP1 staining (and did not bind to γ -adaptin; Fig. 3c). Thus epsin 1 mutants may be more specific inhibitors of AP2-mediated, coated-vesicle transport, whereas AP180 mutants would be predicted to affect all clathrin-mediated budding events.

Role of helix 0 in membrane curvature

Tubulation of liposomes has been observed with a number of proteins^{17–19}. In the cases of dynamin, amphiphysin and endophilin the tubulation can be attributed at least in part to self-oligomerization. Electron microscopy observations revealed that amphiphysin (Fig. 1b) and dynamin can both form tubular extensions from liposomes. With epsin, these intermediates were not seen, implying that it does not tubulate by means of oligomerization. In the case of epsin ENTH domain, we were not able to detect oligomerization by crosslinking on liposomes or by gel-filtration or ultracentrifugation, in the absence or presence of saturating concentrations of head group, and at protein concentrations up to 0.5 mM (not shown). The protein clearly behaved as a monomer, so we conclude that monomeric interactions cause changes in membrane curvature.

We have noted the formation of an amphipathic helix 0 on binding of $\text{Ins}(1,4,5)\text{P}_3$ to the ENTH domain (Fig. 2). The hydrophobic outer surface of helix 0 was targeted for mutagenesis. Mutants of residue L6 to E, Q, H and W showed less binding to liposomes, implying a role for helix 0 in membrane interactions

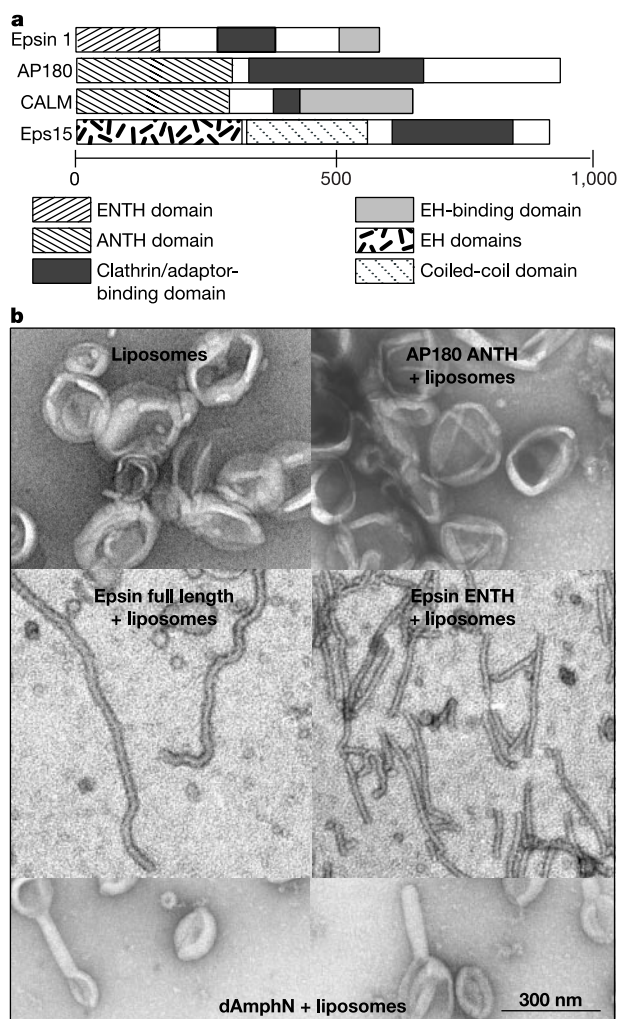


Figure 1 Epsin 1 ENTH domain tubulates liposomes. **a**, Modular arrangement of endocytic proteins. The epsin family can be recognized by the presence of an N-terminal lipid-binding ENTH domain and a clathrin/adaptor-binding domain. The ENTH domain is homologous over the first 150 residues to the ANTH domains of AP180, CALM and HIP1, although the lipid-binding residues are very different (see Fig. 2d). The ENTH domain is followed by ubiquitin-interacting motifs. The adaptor-binding motifs in epsin 1 (eight DPW motifs) and DPF-like motifs in other endocytic proteins bind to the appendage domains of the AP2 complex²⁵. NPF motifs at the C terminus of epsin 1 interact with the Eps15 homology (EH) domain of Eps15 (refs 7–9). The scale bar is in amino acid residues. **b**, Electron microscopy of liposomes in the presence of the domains indicated. dAmphN, *Drosophila* amphiphysin N-terminus²⁶. The outer diameter of tubules formed with epsin ENTH domain was $15 \pm 1 \text{ nm}$, that of tubules formed with full-length epsin was $19 \pm 3 \text{ nm}$, and that in the presence of dAmphN was $46 \pm 2 \text{ nm}$.

(Fig. 3b). Furthermore, the ability of these mutants to tubulate liposomes was related to hydrophobicity of the substituted residue (Fig. 5). Thus L6Q with its hydrophobic and polar component bound to liposomes but failed to tubulate them. L6H tubulated liposomes (although less markedly than wild type), whereas L6W with its larger hydrophobic surface led to extensive tubulation and vesiculation. Although the L6E mutant still bound to short chain lipids in solution (Fig. 3a), the negative charge on the outer surface of this helix probably repels the membrane. From this we conclude that the hydrophobic outer surface of helix 0 is crucial for the induction of membrane curvature.

We tested these mutants in full-length epsin for their distribution in COS cells (Fig. 4, fourth row and data not shown) and found that all the L6 mutants still localize in puncta just like wild-type epsin. Consistent with epsin targeting being predominantly due to binding

of PtdIns(4,5)P₂ head groups, we found that the L6E and L6Q mutants can indeed bind to short chain lipid diC₈PtdIns(4,5)P₂ and Ins(1,4,5)P₃ with affinities close to wild type (Fig. 3a).

Membrane invagination coupled to clathrin polymerization

To study the effects of epsin on clathrin recruitment in conjunction with lipid binding we used lipid monolayers containing PtdIns(4,5)P₂ (Fig. 6). We previously introduced this methodology for visualization and dissection of clathrin assembly by electron microscopy¹³, and we found here that AP180 recruited clathrin to the monolayer where it polymerized into patches of defined size (see also Fig. 6f). These patches are approximately the diameter of coated vesicles in the brain, consistent with the use of brain isoforms of proteins and brain-derived clathrin. This points to a function of AP180 in limiting vesicle size (see also refs 13, 20, 21). We showed

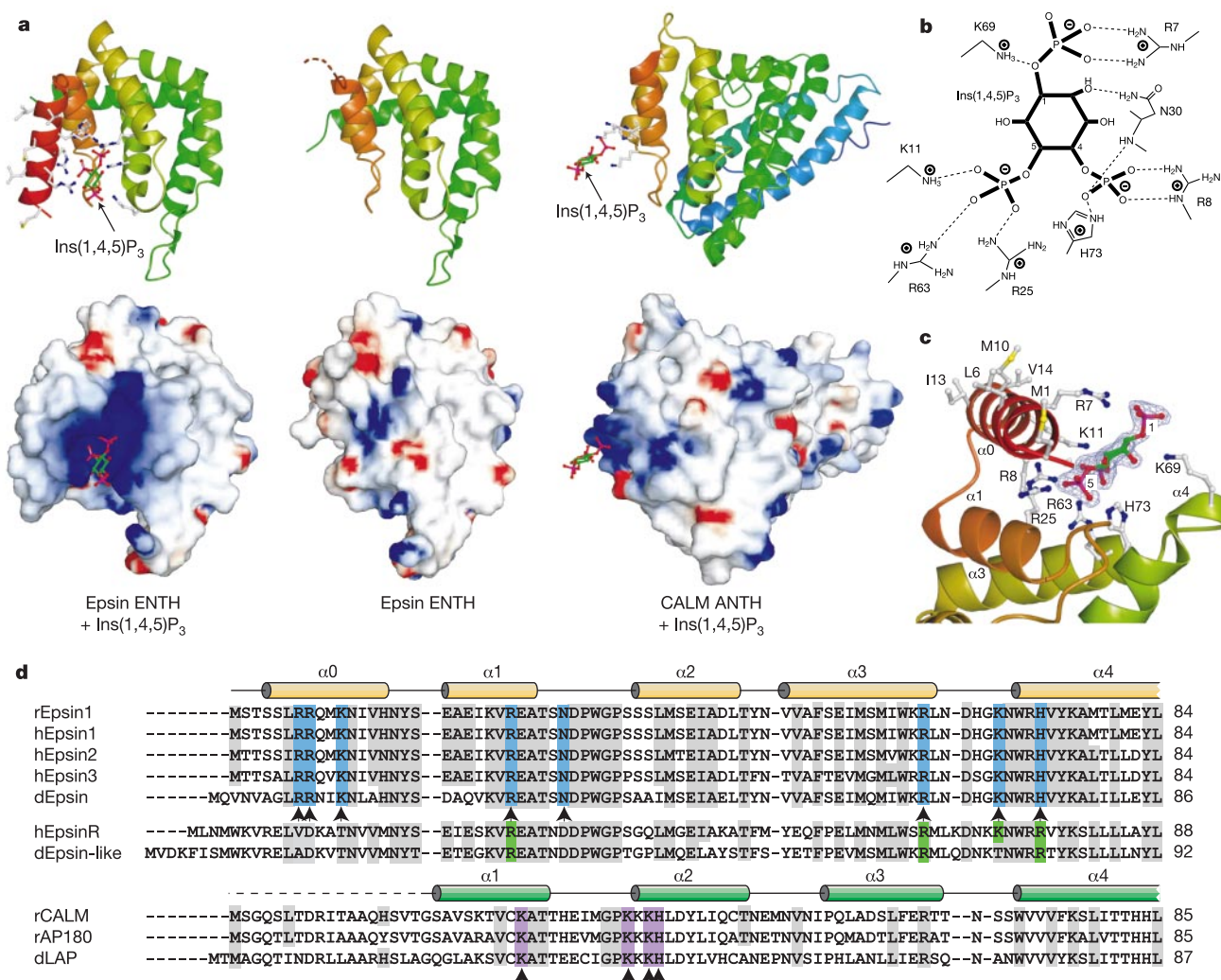


Figure 2 Structure of epsin ENTH bound to Ins(1,4,5)P₃. **a**, Ribbon diagrams of epsin ENTH bound to Ins(1,4,5)P₃ (Protein DataBank (PDB) accession number 1H0A), and for comparison the previous structures of epsin ENTH solved in the absence of Ins(1,4,5)P₃ (PDB 1EDU¹⁴) and CALM ANTH bound to diC₈PtdIns(4,5)P₂ (PDB 1HFA¹³). The structures are coloured red to blue from N- to C-termini, with corresponding helices having the same colour. Surface electrostatic potentials (red = -10 kT e⁻¹; blue +10 kT e⁻¹) of each structure are shown below. Ins(1,4,5)P₃ binds to CALM on a positively charged surface not present in epsin. **b**, Schematic diagram of the interactions responsible for binding the Ins(1,4,5)P₃ molecule. **c**, Close-up view of the Ins(1,4,5)P₃ binding site, showing the residues responsible for interaction with the ligand. The hydrophobic residues L6, M10 and I13 exposed on formation of helix 0 are also marked. The structure shows that lipid

binding and an interaction of helix 0 with the bilayer could happen simultaneously. The electron density for the ligand is shown, contoured at 0.168 e⁻³. **d**, Sequence alignments comparing the lipid-binding residues of all the epsin family members with corresponding residues from the ANTH domains of AP180, CALM and LAP (the *Drosophila* AP180 homologue). Critical residues for Ins(1,4,5)P₃ binding to epsin 1 are coloured in blue and are conserved in epsins 1, 2 and 3 and in *Drosophila* epsin (*liquid facets*). The lipid-binding residues are not well conserved in epsinR/*Drosophila* epsin-like (see residues coloured green), suggesting a different lipid specificity of this epsin. The epsin Ins(1,4,5)P₃-binding residues are not conserved in AP180 and CALM, where a different set of residues have been identified as being involved in Ins(1,4,5)P₃ binding, coloured in purple¹³. h, human; d, *Drosophila*; r, rat.

that these patches are flat lattices, whereas the presence of the AP2 complex gave limited invagination¹³. Epsin, similar to AP180, was able to recruit and promote clathrin polymerization on the monolayer (Fig. 6a, d, e; see also <http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/EM>), but the clathrin was less uniformly polymerized than in the presence of AP180, as seen from the high background of clathrin triskelia (Fig. 6d, compare with b and c) and the less efficient recruitment of clathrin to liposomes by epsin (data not shown). Where there was clathrin polymerization by epsin, it was generally more extensive than with AP180, and showed clear signs of invagination. Pentagons can be resolved in the clathrin patches (implying curvature) and the three-dimensional shape of the clathrin-coated buds can be visualized using stereo images (Fig. 6d). Shadowing the clathrin patches with platinum also showed that they are invaginated; however, this process destroyed most of these structures (not shown). Even without shadowing we frequently found footprints (both large and small) where the

invaginations have been washed off (see left of panel c). We also found this process at an intermediate stage of partial detachment (see right of panel b). Neither the R63L H73L nor the L6E mutants formed clathrin-coated invaginations (data not shown). These observations provide clear evidence of a role for epsin in membrane invagination together with the polymerization of clathrin.

Epsin was originally identified as a principal binding partner of

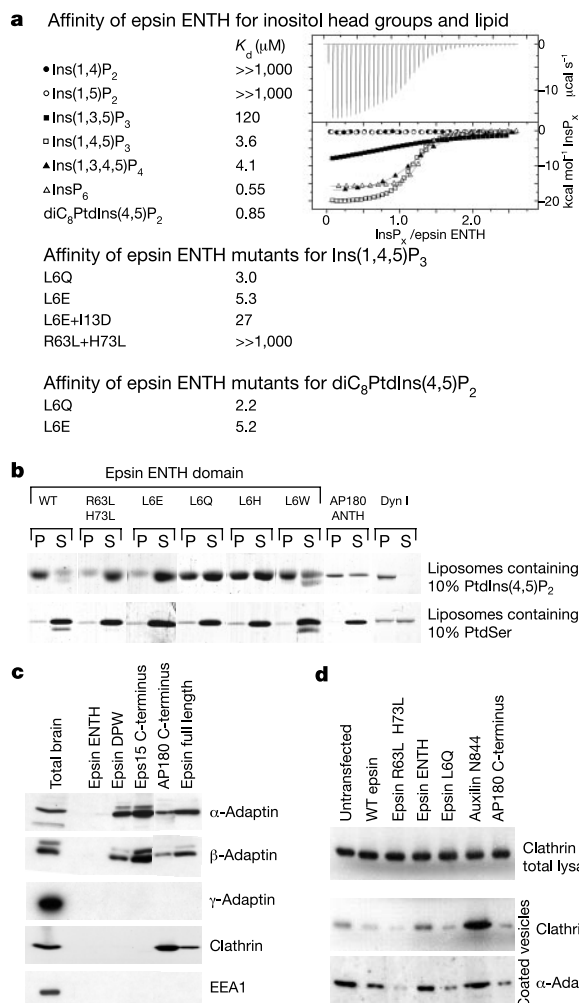


Figure 3 Binding of epsin ENTH and mutants to phosphoinositides. **a**, Dissociation constants (K_d) for wild-type and mutant epsin ENTH domains with inositol phosphates and the short chain lipid diC₈PtdIns(4,5)P₂ determined by calorimetric titration at 10 °C. **b**, Liposome pelleting assays. Only liposomes containing 10% PtdIns(4,5)P₂ efficiently sedimented epsin ENTH, whereas the Ins(1,4,5)P₃ binding site mutant (R63L H73L) was not pelleted. Dyn I, dynamin 1; P, pellet; S, supernatant. **c**, Binding partners in rat brain extract for the indicated proteins. **d**, Purified clathrin-coated vesicles from RPMI1788 B cells transiently transfected with the indicated proteins. Clathrin in the total lysates is shown as a control for cell numbers used. Reduced numbers of clathrin-coated vesicles are present in cells overexpressing full-length epsins.

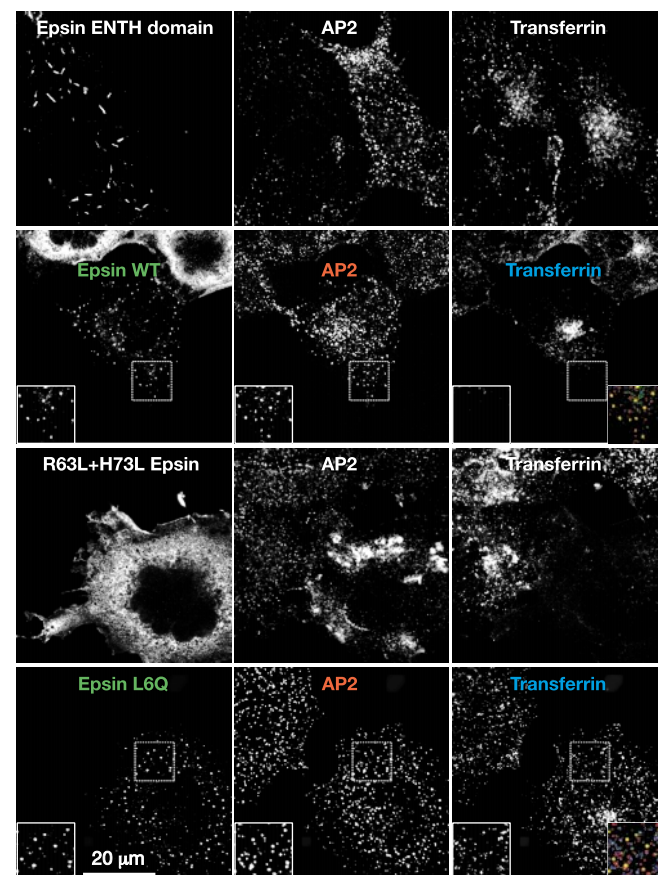


Figure 4 Effect of full-length epsin and mutants on the distribution of the AP2 complex in COS-7 cells visualized by sequential imaging with confocal microscopy. The three columns show the distribution of transfected epsin proteins (left, anti-Myc tag), endogenous AP2 (middle, AP6 antibody) and transferrin uptake (right). A strong perinuclear staining of transferrin is indicative of wild-type endocytosis. Selected enlargements of cells are shown (bottom left corner) to illustrate the co-localization of epsin with AP2, with a merged image in the right of the transferrin column. For co-localization with other endocytic proteins see <http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/IF>

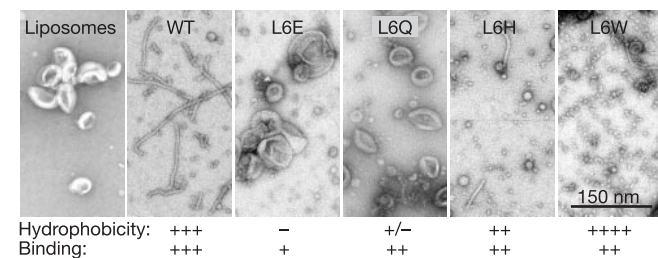


Figure 5 Mutations of L6 on helix 0 affect the ability of epsin to tubulate liposomes. Total bovine brain liposomes (Folch extract) were incubated with 40 μM mutant and wild type (WT) epsin ENTH domains, negatively stained and visualized by electron microscopy. Hydrophobicity of the mutant residue and relative levels of liposome binding (taken from Fig. 3b) are shown.

Eps15, which has been localized to the edge of a forming coated pit²². Therefore epsin may also be targeted to this region where the need for curvature-forming molecules will be greatest. This implies that epsin will not be needed to maintain curvature once the clathrin lattice is laid down and indeed we have previously shown that AP2 facilitates curvature of the lattice on membranes²³. Our results also point to the cooperativity between AP2 and epsin recruitment to membranes, as we found that the localization of the R63L H73L mutant is cytoplasmic, and AP2 staining is no longer punctate

(leading to an inhibition of transferrin endocytosis). Thus membrane invagination and cargo recruitment may go hand-in-hand. Epsin 1 is not enriched in mature coated vesicles of the brain (I.G.M. and H.T.McM., unpublished observations; see also ref. 9), and on liposomes it forms narrower tubules than those formed by amphiphysin or a dynamin. Thus if epsin were finally concentrated at the neck of the budding vesicle, then the narrowing of the neck may provide a weak point for the separation of the vesicle from the parent membrane on GTP hydrolysis of dynamin.

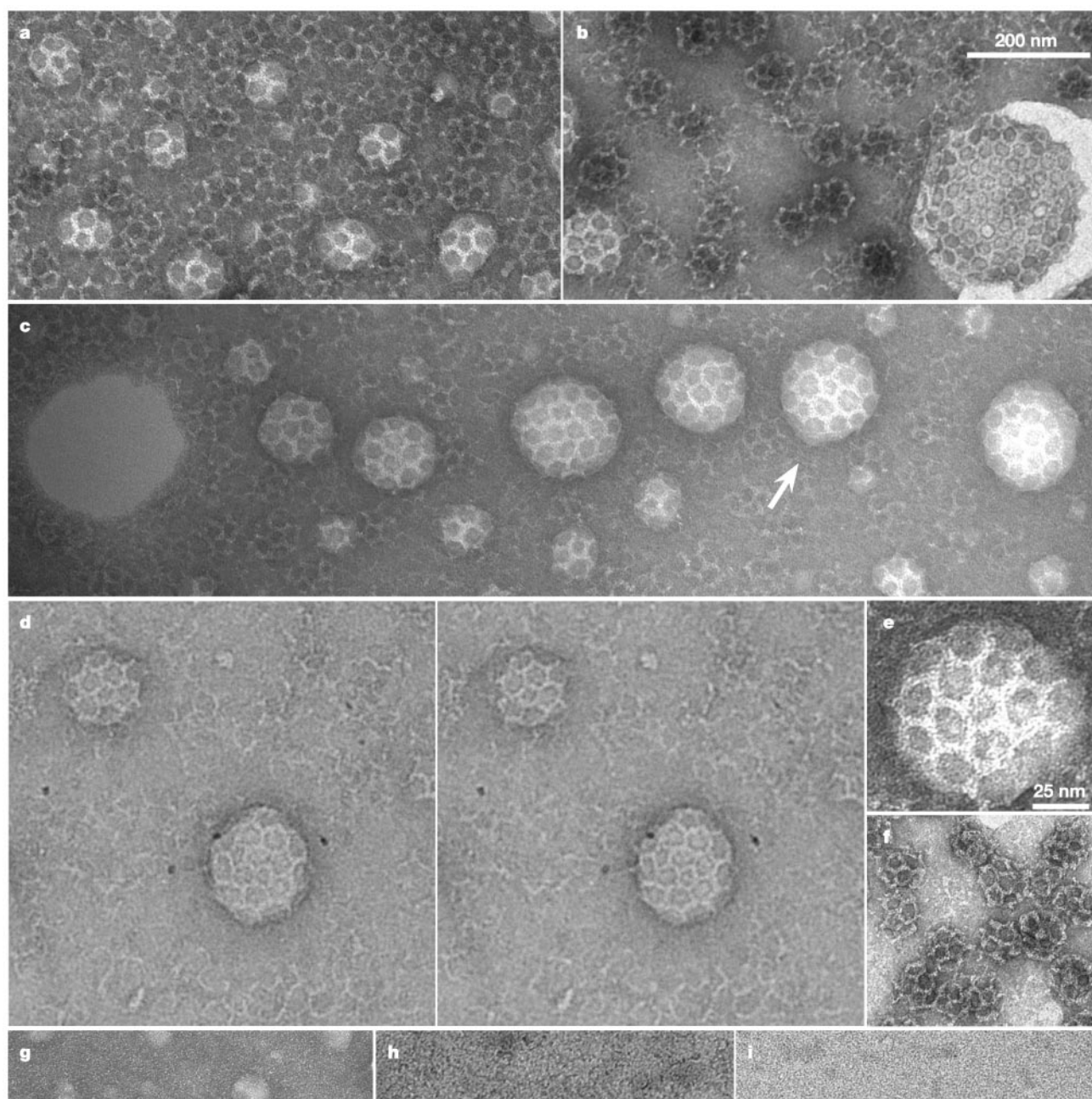


Figure 6 Clathrin recruitment to lipid monolayers by epsin. **a**, Epsin recruits clathrin to lipid monolayers containing 10% PtdIns(4,5)P₂ and stimulates clathrin lattice assembly. The irregular lattices contain both pentagons and hexagons and therefore are slightly raised from the surface of the monolayer. The background lattices are irregular and variable in size and shape. **b**, **c**, In the presence of epsin and AP180, invaginated assemblies are still formed, but the background lattices show a more regular structure reminiscent of the lattices formed when AP180 and clathrin alone are incubated with the monolayers (see **f**). The assemblies contain pentagons and occasionally heptagons (see

arrow). We presume that empty circular profiles in the monolayer result from assemblies that have been sheared off. **d**, Stereo image of a section of monolayer incubated with epsin and clathrin. The assemblies clearly protrude from the surface of the monolayer. **e**, Close-up view of an epsin and clathrin assembly. **g–i**, Control experiments with monolayers. **g**, epsin ENTH and clathrin; **h**, epsin alone; **i**, clathrin alone. The scale bar in **b** applies to all panels except **e**. A gallery of high-resolution images is available at <http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/EM>.

So what is the role of epsin in endocytosis? We have shown that epsin causes a strong degree of membrane curvature and tubulation, even fragmentation of membranes with a high PtdIns(4,5)P₂ content. We envisage that epsin binding to membranes facilitates their deformation by insertion of helix 0 into the outer leaflet of the bilayer, pushing the head groups apart. This would reduce the energy needed to curve the membrane into a vesicle, making it easier for the clathrin cage to fix and stabilize the curved membrane (see also ref. 24). Thus our experiments point to a pioneering role for epsin in vesicle budding as it provides both a driving force and a link between membrane invagination, clathrin polymerization and AP2 complex recruitment. □

Methods

See Supplementary Information for the complete description of the Methods (Protein expression and purification; Transfections; Crystallisation and structure determination; Isothermal titration calorimetry).

Liposome tubulation, sedimentation and electron microscopy

Synthetic liposomes contained 10% cholesterol, 40% phosphatidylethanolamine, 40% phosphatidylcholine, and 10% of the test lipid (in most cases phosphatidylinositol(4,5)bisphosphate). Bovine brain lipids (Folch Fraction 1, which contains approximately 10% phosphatidylinositol lipids) were purchased from Sigma (B1502). Liposomes were resuspended at 1 mg ml⁻¹ in 50 mM HEPES, pH 7.4, 120 mM NaCl and extruded through a 0.4-μm cyclopore filter. For electron microscopy (EM), proteins (4 μM) were incubated with liposomes (0.1 mg ml⁻¹) for 1 min and absorbed onto a glow-discharged carbon-coated EM grid and stained with uranyl acetate before visualisation. For tubulation and sedimentation assays GST tags have been cleaved from the proteins but the presence of an N-terminal GST tag did not prevent either the tubulation of Folch liposomes by epsin or sedimentation. Hydrophobicity of the L6 mutants was determined using the Hopp–Woods scale²⁷. For spin assays liposomes and protein (final volume of 40 μl) were incubated for 10 min at room temperature and then spun at 30,000 r.p.m. for 10 min in a Beckman TLA 100 rotor.

Lipid monolayers (same composition as for synthetic liposomes) were formed on the surface of a buffer droplet in a Teflon block and protein(s) of interest were introduced into the buffer¹³. A carbon-coated gold EM grid was placed on the monolayer, incubated at room temperature for 60 min, and then removed and stained with uranyl acetate.

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Competing interests statement

The authors declare that they have no competing financial interests.

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The harlequin mouse mutation down-regulates apoptosis-inducing factor

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Harlequin (*Hq*) mutant mice have progressive degeneration of terminally differentiated cerebellar and retinal neurons. We have identified the *Hq* mutation as a proviral insertion in the apoptosis-inducing factor (*Aif*) gene, causing about an 80% reduction in AIF expression. Mutant cerebellar granule cells are susceptible to exogenous and endogenous peroxide-mediated apoptosis, but can be rescued by AIF expression. Overexpression of AIF in wild-type granule cells further decreases peroxide-mediated cell death, suggesting that AIF serves as a free radical scavenger. In agreement, dying neurons in aged *Hq* mutant mice show oxidative stress. In addition, neurons damaged by oxidative stress in both the cerebellum and retina of *Hq* mutant mice re-enter the cell cycle before undergoing apoptosis. Our results provide a genetic model of oxidative stress-mediated neurodegeneration and demonstrate a direct connection between cell cycle re-entry and oxidative stress in the ageing central nervous system.

Subtle alterations in mitochondrial or antioxidant function from mutations in genes encoding either mitochondrial- or nuclear-encoded mitochondrial proteins can result in abnormal energy production, generation of reactive oxygen species (ROS), and result in programmed cell death. Mitochondrial dysfunction has been implicated in numerous neurodegenerative disorders, including amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and retinal degeneration (reviewed in ref. 1). Furthermore, the late onset observed in the sporadic forms of these disorders is consistent with age-related oxidative stress. In agreement, accumulation of ROS-damaged proteins, lipids and nucleic acids in regions of neuron death have been observed (reviewed in ref. 2). Although oxidative stress has an important role in the pathogenesis of several common human neurodegenerative disorders, the factors causing an imbalance that favour ROS generation over antioxidant defences, and the molecular events that result from oxidative stress leading to neuronal death, remain unclear.

Disturbances in cell cycle regulation have been implicated in neurodegenerative disorders. Abnormal expression of cell cycle markers and *de novo* DNA synthesis in hippocampus, cholinergic basal forebrain and neocortical association areas distinguish post mortem tissue from Alzheimer's disease patients from that of non-Alzheimer's disease subjects^{3–5}. Furthermore, increased levels of cell-cycle-associated proteins are associated with neurodegeneration in patients with Pick's disease and intractable temporal lobe epilepsy⁶. A loss of cyclin-dependent kinase inhibitor expression and an increase in the expression of components of the cell cycle machinery are seen in brains of patients with cerebral ischaemia and experimental ischaemic animal models^{7,8}.

Aberrant cell cycle re-entry of non-terminally differentiated granule cells is associated with target-related granule cell death in the mouse cerebellar mutants staggerer (*Rora*^{sg}; *sg*) and lurcher (*Grid2*^{Lc}; *Lc*), and with degeneration of chronically depolarized cerebellar granule cells in weaver (*Kcnj6*^{wv}; *wv*) mutant mice^{9,10}. Dorsal root ganglion neurons in mice lacking neurotrophin 3 (*Ntf3*) and in cisplatin-treated rats also re-enter the cell cycle before their death^{11,12}. Furthermore, transgenic expression of SV40 T antigen in terminally differentiated neurons causes cell death^{13,14}. Although it is clear that a wide range of primary insults can result in the cell cycle re-entry of different neurons, and that cell cycle abnormalities can lead to neuronal death, the cascade of molecular events that ultimately force neurons back into the cell cycle have yet to be identified.

We have used a positional cloning strategy to identify the molecular defect in a late-onset neurodegenerative mouse model, harlequin (*Hq*). Although the X chromosome-linked *Hq* mutation was originally identified by a complete lack of hair in hemizygous males (*Hq/Y*) and homozygous females (*Hq/Hq*)¹⁵, ataxia and loss of cerebellar neurons have been reported also in aged mutant mice¹⁶. We report here that the *Hq* mutation is a murine ecotropic proviral insertion in the gene encoding apoptosis-inducing factor (*Aif*), also known as programmed cell death 8 (*Pdcd8*). AIF is a flavoprotein with a pyridine nucleotide-disulphide oxidoreductase domain, a domain found in several bacterial hydrogen peroxide scavengers^{17–20}. Normally, AIF is confined to the mitochondrial intermembrane space; however, during conditions that induce apoptosis, AIF translocates to the cytoplasm and nucleus¹⁸. Microinjection of AIF into the cytoplasm of normal cells results in hallmarks of apoptosis including the dissipation of mitochondrial transmembrane potential and chromatin condensation, which occur in a manner that is apparently caspase- and Bcl2-independent¹⁸. In addition, embryonic stem cells with a targeted null mutation in *Aif* have increased resistance to serum starvation, further defining the apoptogenic role of AIF. However, chimaeric mice could not be generated from these targeted cells, leaving the role of AIF in the adult mouse unknown²¹.

We demonstrate here a vital role for AIF in neuron survival in the ageing mouse brain. Reduced levels of AIF lead to an increase in peroxide sensitivity and to progressive increases in ROS-induced damage in neurons. Furthermore, we demonstrate that terminally differentiated neurons in the cerebellum and retina of animals in which AIF is downregulated undergo unscheduled cell cycle re-entry, providing a molecular mechanism by which free radical damage can lead to neuronal death.

Cerebellar neuron loss in *Hq* mutant mice

We histologically examined cerebella from pre- and post-ataxic mutant mice. Before three months of age, cerebella of hemizygous or homozygous mutant mice were indistinguishable from those of littermate controls (Fig. 1a, c). At four months, however, many pyknotic granule cell nuclei were observed, and by seven months, mutant cerebella were much smaller than age-matched littermate controls (Fig. 1b, d). Granule cells were preferentially lost in the caudal (from lobules 6–10) vermis and hemispheres, although some granule cells in the rostral portion of the cerebellum also

degenerated (Fig. 1d). By 12 months of age, most of the granule cells in the caudal half of the cerebellum were lost (data not shown). In contrast, no loss of cerebellar cells were observed in heterozygous mice, even at 26 months of age (data not shown).

To investigate further the nature of neuron loss in *Hq* mutant mice, TdT-mediated dUTP nick end labelling (TUNEL) staining—which detects nicked DNA, characteristic of apoptotic cells—and electron microscopic analysis were performed. TUNEL-positive granule cells were observed in four-month-old *Hq* mutant mice but not in littermate controls (Fig. 1e and data not shown). In

addition, electron microscopic analysis demonstrated that dying granule cells had hallmarks of apoptosis including nuclear condensation and blebbing (Fig. 1f).

Purkinje cells in *Hq* mutant mice also degenerate; however, unlike the early-onset cerebellar degeneration mutants reported to date in which granule cell death seems secondary to the loss of Purkinje cells (reviewed in ref. 22), death of granule cells in *Hq* mutant mice occurs before Purkinje cell loss. At four months of age when many pyknotic granule cell nuclei are observed, almost all Purkinje cells are still present (Fig. 1g, h). However, by seven months, many

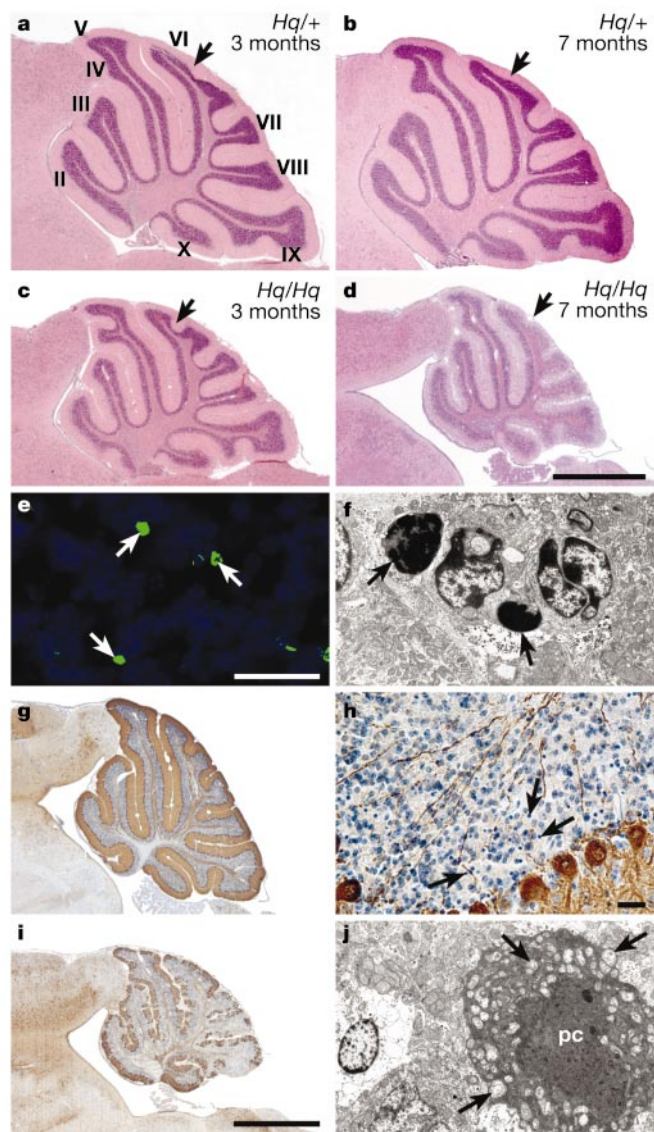


Figure 1 Progressive ataxia in *Hq* mutant mice is correlated with the loss of cerebellar granule and Purkinje cells. **a–d**, Haematoxylin and eosin stained sagittal sections from *Hq/Hq* (**c, d**) or littermate controls (**a, b**) at three months (**a, c**) or seven months (**b, d**) of age. Note the anterior–posterior boundary of granule cell degeneration (arrow) in lobule 6. **e**, TUNEL-stained cells (green) from the cerebellum of a five-month-old *Hq/Y* male (arrows) counterstained with Hoescht 33342 (blue). **f**, Electron micrograph of apoptotic granule cells from a four-month-old *Hq/Y* mouse (magnification $\times 5,000$), showing nuclear condensation (arrow). **g–i**, Sagittal sections from *Hq/Hq* (**g, h**) or *Hq/Y* (**i**) cerebella immunostained with calbindin-D28 antibodies to visualize Purkinje cells and counterstained with haematoxylin (pyknotic granule cells are indicated by arrows in **h**). **j**, Electron micrograph of a necrotic Purkinje cell (pc) from a four-month-old *Hq/Y* mouse (magnification $\times 5,000$) showing swollen mitochondria (arrows). Scale bars: 1 mm, **a–d, g, i**; 20 μm , **e, h, j**.

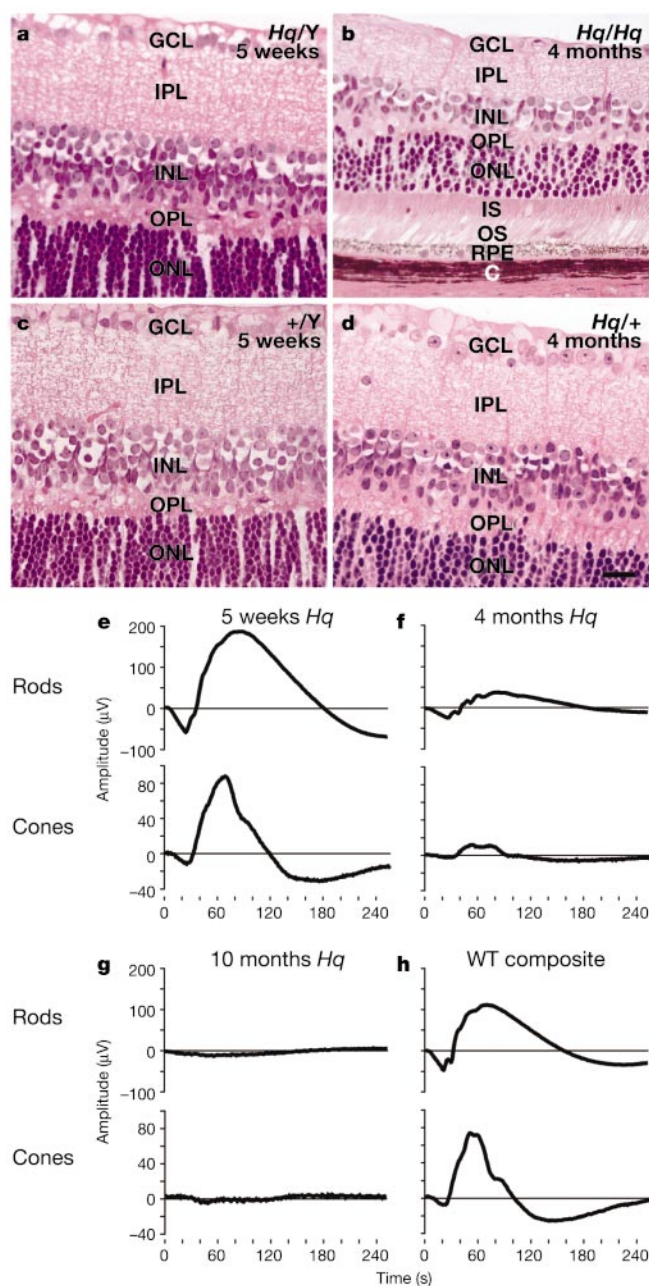


Figure 2 Progressive retinal degeneration in *Hq* mutant mice. **a–d**, Haematoxylin and eosin stained sections from *Hq/Y* (**a**), *Hq/Hq* (**b**) or littermate controls (**c, d**) at five weeks (**a, c**) or four months (**b, d**) of age. Scale bar, 20 μm . GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; IS, inner segment; OS, outer segment; RPE, retinal pigmented epithelium; C, choroid. **e–h**, Electroretinograms of *Hq* mutant mice (**e, f, g**) and a composite of wild-type (WT) controls at all ages (**h**).

Purkinje cells have died and, as with granule cell death, this cell loss is higher in the caudal lobules of the cerebellum (Fig. 1i). Electron micrographs of dying Purkinje cells revealed swollen mitochondria and fragmented cell membranes, suggesting that unlike granule cells, Purkinje cells die by means of necrosis (Fig. 1j).

Progressive retinal degeneration in the *Hq* mutant

Progressive retinal degeneration was observed in ageing *Hq* mutant mice. Differences in retinal morphology were not observed between *Hq* mutants and wild-type control littermates before three months of age (Fig. 2a, c). At three months of age, *Hq/Hq* and *Hq/Y* mice exhibit a loss of ganglion and amacrine cells in the ganglion cell layer of the peripheral retina, whereas all other cell layers remain intact (data not shown). However, in four-month-old *Hq/Hq* and *Hq/Y* mice, cell loss was observed in the ganglion cell layer and the inner and outer nuclear layers (Fig. 2b, d). Cell loss continues as the mice

age, and by 11 months cell loss is very apparent in all cell layers of the retina (data not shown). Furthermore, the inner and outer plexiform layers, containing the axons and dendrites of these neurons, have thinned out considerably. Retinal neuron loss was not observed in aged *Hq/+* mice (data not shown).

Electroretinography (ERG) performed on *Hq* mutant mice and littermate controls confirmed our histological findings. Full-field ERG was performed as previously described²³. *Hq* mice at five weeks of age show normal rod and cone responses (Fig. 2e). However, by four months of age, both the rod and cone ERG responses in *Hq* mutant mice diminish, although the shapes of the curves are normal (Fig. 2f). By 10 months, both the rod and cone responses are completely abolished in *Hq* mutant mice (Fig. 2g), whereas control littermates show normal ERG responses (Fig. 2h). Therefore, both clinically and histologically, *Hq* mutant mice exhibit late-onset retinal degeneration.

The *Hq* mutation disrupts *Aif*

To genetically map the position of the *Hq* mutation, we analysed 1,152 offspring from an intersubspecific backcross. This analysis defined the *Hq* critical region to 0.61 ± 0.11 cM (7 recombinants, 1,152 meioses) between *DXMit48* and *DXMit82* (Fig. 3a). Yeast artificial chromosomes (YACs) containing these markers were identified from the Whitehead/MIT Center for Genome Research set. A 610-kilobase YAC (Y161_B_12) containing both flanking markers, and thus spanning the interval, was identified.

The human genomic sequence homologous to this region of the mouse X chromosome contained several potential *Hq* candidate genes, whose mouse orthologues are: *Elf4*, an ETS-domain transcription factor; *Aif*; *Rab33a*, a member of the RAS family of small GTPases; *Slc25a14*, a brain mitochondrial carrier protein; as well as the mouse orthologues of a putative zinc finger protein (GenBank accession number AL133204) and a putative RNA-binding protein (GenBank accession number AL050405). Polymerase chain reaction (PCR) analysis confirmed that these genes were present on YAC161_B_12, and analysis of critical recombinants for single nucleotide polymorphisms present in the *Slc25a14* 3' untranslated region and intron 16 of the *Aif* gene, demonstrated that these genes co-segregate with the *Hq* mutation. However, neither expression differences nor complementary DNA polymorphisms between *Hq* mutant and wild-type cerebellar RNA were detected in transcripts from the *Elf4*, *Rab33a*, *Slc25a14*, the zinc finger or RNA-binding protein-encoding genes by northern analysis and sequencing of PCR with reverse transcription (RT-PCR) products, respectively. In contrast, *Aif* transcript and protein levels were reduced by 80% relative to wild-type levels in the cerebellum, the remainder of the brain, eyes, kidney, muscle, lungs and liver of pre-ataxic, one-month-old *Hq* mutant mice (Fig. 3b, see also Supplementary Information Fig. 1; data not shown). However, *Aif* transcript and protein levels in tissues of *Hq/+* mice were only slightly decreased from wild-type levels, consistent with the lack of cell death in heterozygous females.

To further test *Aif* as a candidate gene for the *Hq* locus, its cerebellar and retinal expression was examined. Consistent with the pattern of neuron loss in mutant mice, AIF expression was seen in both granule and Purkinje cells of the wild-type cerebellum and in all cell layers of the retina (Supplementary Information Fig. 2). However, analysis of RT-PCR products from *Hq* mutant brain RNA using *Aif* primers revealed no sequence differences from wild-type message and that, like wild type, both splice forms of *Aif* exon 2 are present in *Hq* mutant tissues.

Introns 2–16 were amplified by PCR from both mutant and wild-type genomic DNA, and no size differences between *Hq* mutants and wild-type controls were observed. However, multiple restriction-fragment length polymorphisms between *Hq/Y* and *+/Y* genomic DNA were detected by Southern blot analysis with a probe from intron 1 (Supplementary Information Fig. 3). Notably,

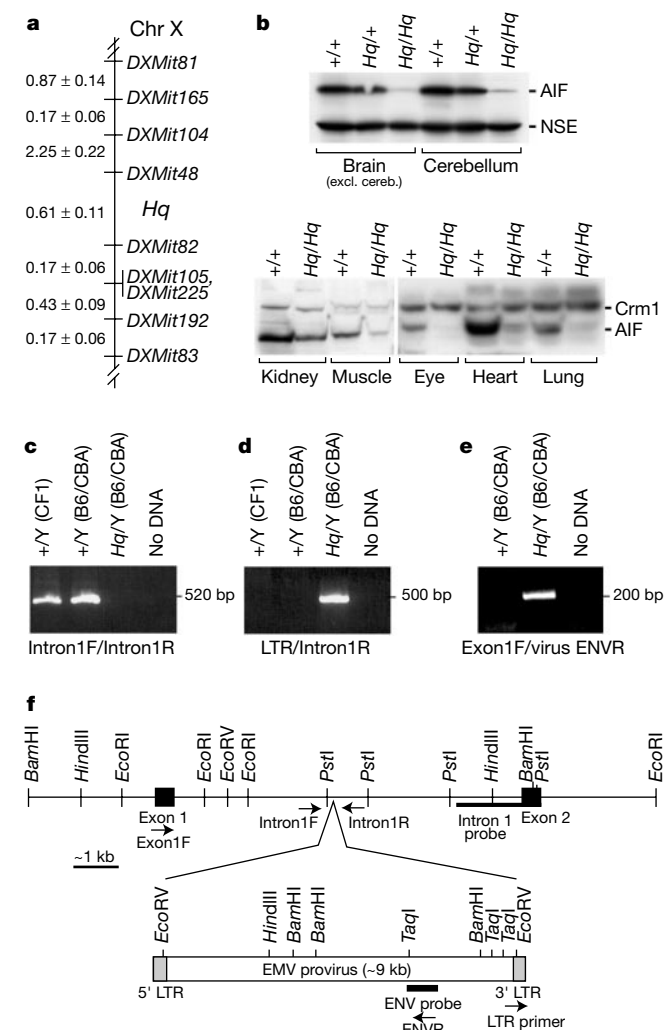


Figure 3 The *Hq* mutation is an ecotropic proviral insertion in the *Aif* gene. **a**, Chromosomal location of the *Hq* mutation (values are in cM). **b**, Western blot of protein from several tissues of wild-type, *Hq/+* and *Hq/Hq* mice with neuron-specific enolase (NSE) and Crm1 as loading controls. **c**, PCR on genomic DNA from wild-type (CF1 and B6CBA) or *Hq/Y* mice with *Aif* intron primers (forward (F) and reverse (R)). **d**, PCR on genomic DNA from wild-type or *Hq/Y* mice with a primer made to the U3 region of the C-type murine ecotropic virus LTR (LTR primer) and a reverse primer from intron 1. **e**, RT-PCR reactions using primers corresponding to exon 1 (exon1F) and the viral envelope (ENVR). **f**, Representation of the proviral insertion in the *Hq* mutant mouse. Placement of restriction enzymes in the proviral insertion was based on the murine C-type ecotropic virus (Emv; Genbank accession number U63133).

in *EcoRI*-digested genomic DNA the mutant band was larger than that of the control (16 kb compared with 7 kb, respectively). Murine ecotropic leukaemia proviruses, which are endogenous retroviruses normally present at 1–2 copies per mouse genome, are approximately 8.8 kb in size and do not contain *EcoRI* restriction sites within their genome²⁴. Southern blots were rehybridized with a probe specific for the ecotropic proviral envelope gene²⁵, and bands that were identical in size to those detected with the intron-specific probe were observed in *Hq*, but not wild-type, genomic DNA digested with several different enzymes (Supplementary Information Fig. 4).

Mutant and wild-type genomic DNA was subjected to PCR analysis using pairs of primers designed to produce overlapping PCR products spanning the 7.7-kb intron 1. One primer pair (intron 1 forward/1 reverse), at base pair 3,262 and 3,772 of intron 1, amplified products from control DNAs, but failed to amplify mutant DNA (Fig. 3c). Conversely, PCR reactions using this reverse intron primer and an ecotropic long terminal repeat (LTR)-specific primer positioned 420-bp upstream of the 3' end of the provirus amplified a product from mutant, but not wild-type, DNA (Fig. 3d). Sequencing confirmed this product contained LTR sequence juxtaposed to mouse genomic sequence beginning at base pair 3,432 of intron 1. To determine whether this provirus was unique to *Hq* mutant mice, PCR reactions with both primer pairs were repeated on genomic DNA from 23 inbred strains (see Methods). In all cases, the intron fragment was amplified and the LTR/intron reverse primer pair failed to generate products.

RT-PCR was performed using total RNA from *Hq* mutant and wild-type brains using PCR primers corresponding to exon 1 of *Aif* and in the envelope of the ecotropic provirus—the site of the most commonly used viral splice acceptor²⁶. Products were observed from mutant but not from wild-type control littermates (Fig. 3e). Sequence of these products verified that aberrant splicing occurs between exon 1 of the *Aif* transcript and the inserted provirus. Together, these results demonstrate that the *Hq* genome contains an ecotropic proviral insertion in intron 1 of the *Aif* gene (Fig. 3f). Like many other proviral insertions, this insertion leads to a decrease in gene expression with approximately an 80% reduction in *Aif* messenger RNA and protein levels in *Hq* mutant mice compared with wild-type control levels.

Loss of AIF function leads to oxidative stress

AIF is a mitochondrial oxidoreductase that translocates to the nucleus leading to nuclear condensation and apoptosis¹⁸. In addition to its role in inducing apoptosis, AIF has a potent redox function *in vitro*, which is separable from its apoptogenic activity²⁰. Many oxidoreductases have been shown to have important roles in maintaining intracellular free radical homeostasis (reviewed in ref. 27). Under conditions favouring excess free radical production, lipid peroxidation and oxidative damage of DNA may ensue,

leading to events associated with late-onset neurodegenerative disorders.

We examined antioxidant enzyme levels, lipid peroxidation and DNA oxidative damage in *Hq* and wild-type mice. Owing to the similarity of the oxidoreductase moiety of AIF to bacterial hydrogen peroxide scavengers, we hypothesized that changes in catalase and glutathione levels would occur in *Hq* mutant mice. Catalase is a principal scavenger of hydrogen peroxide in cellular systems (reviewed in ref. 28), whereas glutathione is an essential electron donor for the reduction of hydroperoxides²⁹. In agreement with the above hypothesis, catalase activity is increased in the cerebella of *Hq* mutant mice at both 1 and 3 months of age compared with wild-type levels (Fig. 4a). However, no differences were noted in the remainder of the brain at either one or three months of age. Similar to catalase, total glutathione levels were increased in the cerebella of *Hq* mutant mice at both one and three months of age compared with wild-type levels (Fig. 4b). No differences were observed in total glutathione levels in the remainder of the brain at either one or three months. Western blot analysis of cerebellar extracts of one- and three-month-old *Hq* mutant and wild-type mice showed increases in catalase expression consistent with the increased catalase activity, whereas no differences in the levels of the superoxide anion scavengers Sod1 or Sod2 were observed at either age (data not shown).

Increases in lipid hydroperoxides have been linked to oxidative stress in numerous human neurodegenerative disorders (reviewed in ref. 2). In *Hq* mutant mice, lipid hydroperoxides were increased in both brain and cerebellum at one and three months compared with wild-type mice (Fig. 4c). Increased lipid hydroperoxides were also observed in the hearts of *Hq* mutant mice compared with wild-type controls (Supplementary Information Fig. 5).

Immunofluorescence using an antibody to 8-hydroxydeoxyguanosine (8-OHdG), a principal component of oxidatively damaged DNA, detected positive neurons in both the cerebella and retinas of *Hq* mutant mice, but not of wild-type littermates (data not shown). Reactive cells were observed in the inner granule layer of the cerebellum, and the inner nuclear and ganglion cell layers of the retina, corresponding to areas of widespread cellular loss in the *Hq* mutant mouse (data not shown). Most of the DNA staining positive for 8-OHdG is non-nuclear, consistent with oxidative damage in the mitochondria. 8-OHdG-positive Purkinje cells and photoreceptors of the retinal outer nuclear layer were not observed.

AIF regulates granule cell hydrogen peroxide sensitivity

Recent crystallization studies reveal that the structure of AIF is highly similar to that of glutathione reductase³⁰, a potent antioxidant and scavenger of hydrogen peroxide. Primary granule cell cultures from mutant and wild-type animals were treated with increasing doses of hydrogen peroxide (0, 5, 20, 50 μ M) for 5 h and then stained with propidium iodide to identify dead cells. Significantly more cell death was observed in cultures of *Hq* mutant cells treated with 20 and 50 μ M of hydrogen peroxide (Fig. 5a). No differences between the viability of wild-type and *Hq* granule cells were observed in normal culture conditions.

In vivo, many neuronal types seem to be unaffected by the *Hq* mutation. To further investigate this apparent neuronal cell-type specificity, primary cultures of mutant and wild-type embryonic cortical neurons were treated with increasing concentrations of peroxide. When cortical cultures were treated under the identical conditions used for granule cell cultures (50 μ M for 5 h), we observed no loss of cell viability relative to untreated controls (data not shown). This suggests that cortical neurons in general are more resistant than granule cells to cell death induced by hydrogen peroxide. When wild-type and *Hq* mutant cortical neurons were treated with 100 μ M hydrogen peroxide for 24 h, cell viability was comparable to reported values for similar culture conditions³¹. However, in contrast to granule cells, no difference

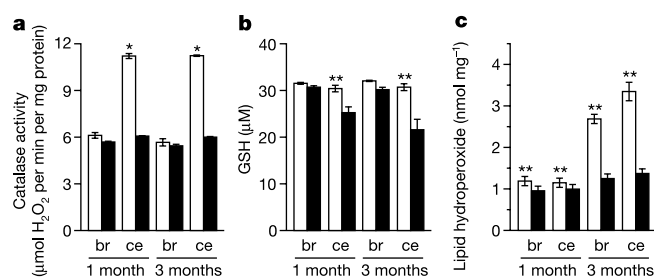


Figure 4 Oxidative-stress-associated responses in *Hq* mutant mice. **a–c**, Graphical representation of catalase activity (**a**), total glutathione (**b**) and lipid hydroperoxides (**c**) in brains (br; without cerebella) and cerebella (ce) of *Hq* mutant mice (open bars) and wild-type control littermates (filled bars). Asterisk, $P < 0.001$; double asterisk, $P < 0.05$.

in viability was noted between mutant and wild-type neurons at any peroxide concentration (Fig. 5d), consistent with the lack of neuronal death in the cortex of aged *Hq* mutant animals (data not shown).

We also examined whether mutant granule cells are more susceptible to endogenous peroxides. It has been suggested that low doses of glutamate cause neuronal excitotoxicity through NMDA (*N*-methyl-D-aspartate) receptors, whereas higher doses of glutamate trigger intracellular oxidative stress through the release of endogenous peroxides³¹. Mutant and wild-type granule cells were treated with 100 μ M and 2 mM glutamate. Cell viability was similar between mutant and wild-type granule cells treated with low concentrations of glutamate, whereas higher doses produced significant increases in *Hq* mutant granule cell death (Fig. 5a). These results demonstrate that downregulation of AIF confers sensitivity to both exogenous and endogenous peroxides.

The sensitivity of mutant and wild-type cerebellar granule cells to non-oxidative stress-mediated cell death was also examined. Previous evidence indicated that embryonic stem cells lacking AIF were more resistant to serum starvation than wild-type cells, but no differences in viability were noted after exposure of these cells to etoposide or staurosporine, which cause DNA cleavage or inhibition of calcium-dependent protein kinases, respectively²¹. Granule cell

cultures were serum starved, or treated with etoposide (50 μ M) or staurosporine (0.5 μ M). After 5 h of serum starvation, significantly less cell death was observed in cultures of mutant granule cells compared with wild type cells, confirming the role of AIF in mediating neuronal apoptosis through growth factor withdrawal (Fig. 5e–g). However, no differences in cell death were observed between *Hq* mutant and wild-type granule cells when subjected to either etoposide or staurosporine for 24 h (Fig. 5e).

To confirm that the peroxide sensitivity of *Hq* mutant granule cells is indeed due to downregulation of AIF, granule cells were infected with murine stem cell retrovirus expressing the *Aif* coding sequence upstream of an internal ribosomal entry site (IRES)–green fluorescent protein (GFP) or the retrovirus without *Aif* sequences³². Three days after infection, cells were treated with hydrogen peroxide and subsequently stained with propidium iodide. The proportion of dead to live cells was determined from both infected (GFP-positive; approximately 50% of each culture) and uninfected (GFP-negative) cells. No differences in cell viability were observed between infected mutant neurons and uninfected wild-type cells at any concentration of peroxide, whereas non-infected *Hq* neurons retained their peroxide sensitivity at 20 or 50 μ M concentrations (Fig. 5h–l). Thus, transduction with wild-type *Aif* rescues the peroxide sensitivity of *Hq* mutant neurons.

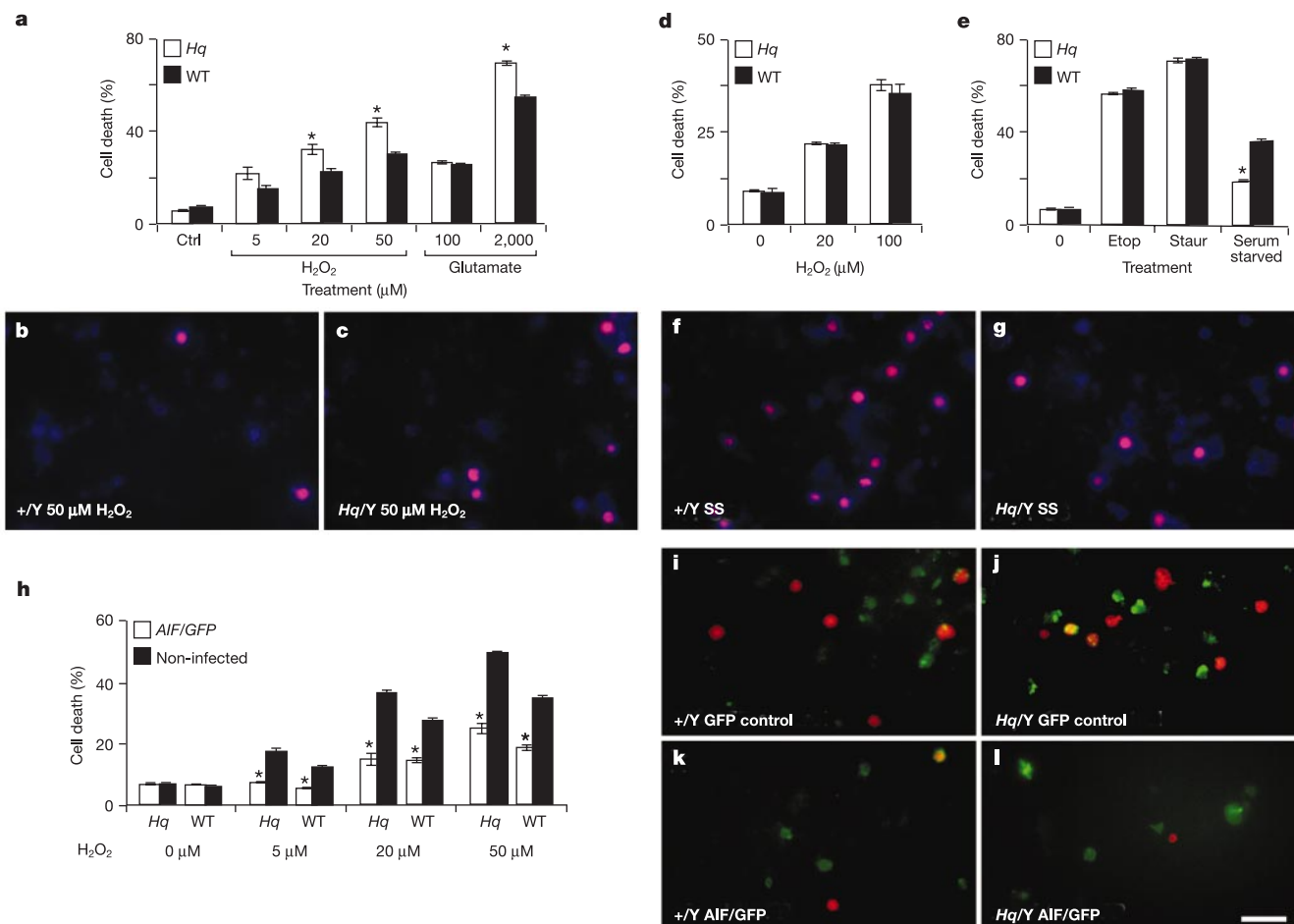


Figure 5 AIF- and peroxide/non-peroxide-mediated cell death in primary neuronal cultures. **a–c**, Wild-type ($n = 4$) (**b**) and *Hq* mutant ($n = 4$) (**c**) granule cell cultures were treated with hydrogen peroxide or glutamate before staining with propidium iodide (red) and Hoechst 33342 (blue), for cell viability and nuclei, respectively. A summary graph of the two treatments is shown (**a**). **d**, Primary cultures of wild-type ($n = 4$) and *Hq* ($n = 6$) cortical neurons were treated with hydrogen peroxide and stained with propidium iodide. **e–g**, Non-peroxide-mediated apoptosis in primary cerebellar granule cells. Etop,

etoposide; SS, serum starved; staur, staurosporine. **h–l**, Retrovirus-infected wild-type (**i**, **k**) and *Hq* mutant (**j**, **l**) cerebellar granule cells infected with an AIF/GFP-expressing virus (**h**, **k**, **l**) or control viruses expressing GFP only (**i**, **j**) before peroxide treatment and propidium iodide (red) staining. To visualize all nuclei, cells were also stained with Hoechst 33342 (not shown). All values are the mean $\pm$ s.e.m. Scale bars, 20 μ m. Asterisk, $P < 0.01$.

Overexpression of AIF in wild-type neurons also resulted in increased peroxide resistance. After peroxide treatment, significantly less cell death was seen in wild-type neurons infected with the AIF-expressing retrovirus than uninfected wild-type neurons (Fig. 5h–l). No difference in viability was observed between uninfected cells and cells of the corresponding genotype infected with control retrovirus expressing GFP but not AIF (Fig. 5i, j, and data not shown). Together, these data suggest that in addition to its apoptogenic role, AIF has antioxidant activity, particularly as a peroxide scavenger.

Cell cycle re-entry by oxidatively stressed neurons

Although oxidative stress has been observed in neurons from patients with several human neurodegenerative disorders, the means by which such stress leads to cell death remain poorly understood. Similarly, cell cycle abnormalities have been reported in neurons in brain regions undergoing degeneration, yet the mechanisms causing cell cycle re-entry of terminally differentiated neurons are unknown. Because oxidative stress has been associated with cell cycle re-entry and abnormal cell cycle checkpoint function

(reviewed in ref. 33), we investigated whether cell cycle control is disrupted in *Hq* mutant neurons.

Hq/Hq mice and *Hq/+* littermate controls were injected with bromodeoxyuridine (BrdU) at 3, 4, 5, 7, 9 and 12 months of age to mark cells that have undergone DNA synthesis. Mice were killed 20 h after injection, and immunohistochemical analysis of 20 adjacent midline sagittal sections was performed. Virtually no labelled granule cells were found in *Hq/+* littermate controls; however, many BrdU-positive granule cells were seen in *Hq/Hq* cerebella (Fig. 6a, b). Aberrant cell cycle re-entry was further confirmed by immunofluorescence with antibodies against proliferating cell nuclear antigen (Pcna) or Cdc47—proteins that are expressed during S phase through the late G2 phase of the cell cycle, but not in quiescent cells (data not shown)^{34,35}. As observed in the BrdU studies, many granule cells in the cerebella of older *Hq/Hq* females, but not cells from aged-matched heterozygotes, were reactive with these antibodies, further confirming cell cycle misregulation in mutant cells (data not shown). To confirm that cycling cells were indeed granule cells, co-immunofluorescence experiments using antibodies to Pcna and GABA_A receptor alpha 6 (GABARA6)—a protein expressed only by terminally differentiated granule cells in the inner granule layer—and Pcna and the glial marker, glial fibrillary acidic protein (Gfap), were performed. All Pcna-positive cells were also positive for GABARA6 but not Gfap, confirming that cycling cells retain the characteristics of terminally differentiated granule cells (data not shown).

The number of BrdU-positive granule cells steadily increased through seven months of age then began declining, probably due to the extensive loss of many granule cells (Fig. 6b). In addition, more BrdU-positive cells were located in the caudal region of the cerebellum, consistent with our findings of increased cell death in the caudal region of the *Hq* mutant cerebellum (see Fig. 1). However, BrdU-positive Purkinje cells were not observed in *Hq* mutants or littermate controls (data not shown). In summary, these data demonstrate that *Hq* mutant granule cells, but not Purkinje cells, re-enter the cell cycle during the time when degeneration is occurring.

In the retina, BrdU- and Pcna-positive ganglion, amacrine and horizontal cells were observed in *Hq* mutant mice but not in wild-type control littermates (data not shown). Similar to our cerebellar data, abnormally cycling retinal neurons were first detected at about four months of age and numbers had increased at seven months, but then began declining as the total population of ganglion, amacrine and horizontal cells decreased.

Many of the cells that incorporated BrdU had pyknotic, blebbing, or fragmented nuclei typical of cells undergoing apoptosis (Fig. 6a). In agreement with this finding, all mutant granule cells expressing activated caspase 3, an initiator of neuronal apoptosis, were also positive for Cdc47 (Fig. 6c–e). In the retina, as in the cerebellum, Cdc47-positive neurons also expressed activated caspase 3 (Supplementary Information Fig. 6). However, no Cdc47 or caspase-3-positive Purkinje or photoreceptor cells were observed. Furthermore, a subpopulation of Cdc47-positive cells in both the cerebellum and retina was not positive for caspase 3, a finding consistent with previous reports from both mouse and human studies demonstrating that cell cycle re-entry precedes neuronal apoptosis^{9,10,36,37}.

Sections from mutant cerebellum and retina were double-labelled with antibodies against 8-OHdG and caspase 3 (full length or activated). Double-positive cells were observed in ganglion, horizontal and amacrine cells of the *Hq* mutant retina (Supplementary Information Fig. 6 and data not shown). Furthermore, granule cells reactive with both antibodies were noted in the *Hq* mutant cerebellum (data not shown). In both cerebellum and retina, all cells expressing either high levels of caspase 3 or activated caspase 3 were positive for 8-OHdG. No cells positive for either 8-OHdG or caspase 3 were noted in the wild-type retina or cerebellum (data

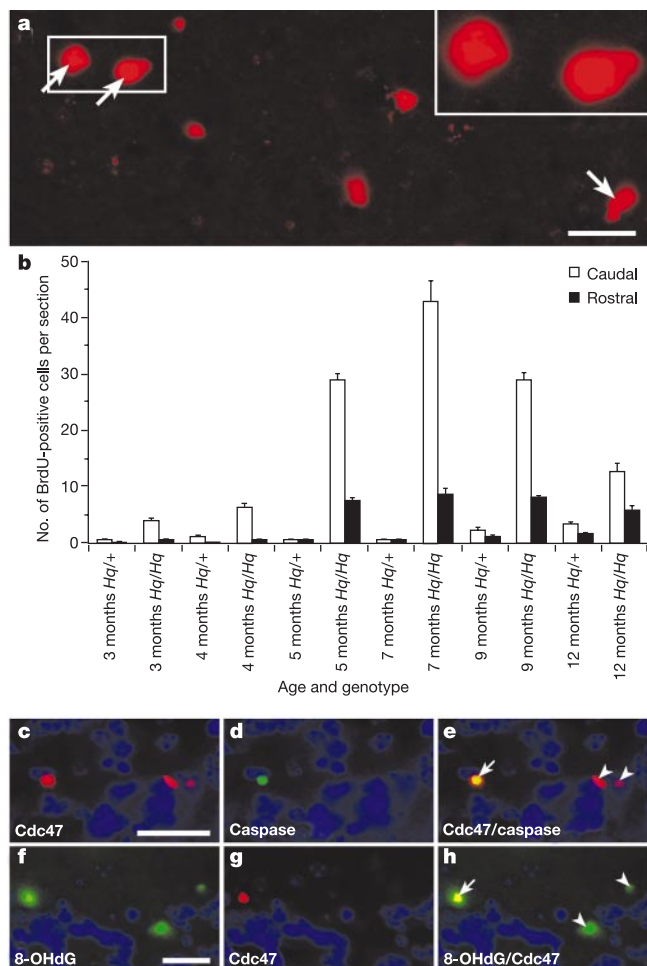


Figure 6 Oxidatively damaged neurons re-enter the cell cycle in *Hq* mutant mice. **a**, BrdU-positive cells in a five-month-old *Hq/Hq* female exhibit blebbing and fragmented nuclei (arrows and insert). **b**, Temporal course of BrdU incorporation in *Hq/Hq* and *Hq/+* females. The average number of positive cells per section is divided into numbers of positive cells in the rostral versus caudal half of the cerebellum (as determined by the boundary depicted in Fig. 1). Error bars indicate standard error. **c–e**, Cdc47 and activated caspase 3 expression in the cerebella of *Hq* mutant mice. **f–h**, Cdc47 and 8-OHdG expression in the cerebella of *Hq* mutant mice. Arrows in **c–h** are used for double-positive cells; arrowheads are used for single-positive cells. Scale bars, 20 μ m.

not shown). As with staining for Cdc47 and caspase 3, a subpopulation of cells in both the retina and the cerebellum of *Hq* mutant mice were positive for 8-OHdG but not caspase 3.

Sections from mutant cerebellum and retina were double-labelled with antibodies against 8-OHdG and Cdc47. All Cdc47-positive cells in both the retina and cerebellum were also reactive with 8-OHdG antibodies (Fig. 6i–k and Supplementary Information Fig. 6). However, 8-OHdG-positive neurons that were not reactive for Cdc47 were also observed, suggesting either a temporal division between oxidative stress and cell cycle re-entry or that not all oxidatively stressed cells re-enter the cell cycle.

Discussion

In the *Hq* mutant mouse, an ecotropic proviral insertion in intron 1 of the *Aif* gene leads to an 80% reduction in mRNA transcripts and protein expression when compared with wild-type levels. The AIF protein, normally present in the inner mitochondrial membrane, contains a pyridine nucleotide-disulphide oxidoreductase subunit similar to plant and bacterial hydrogen peroxide scavengers^{17,19,20}. Furthermore, AIF is structurally similar to glutathione peroxidase, a potent scavenger of peroxides in mammalian cells³⁰. Although studies have determined that mislocalization of the AIF protein leads to nuclear condensation and apoptosis¹⁸, *in vivo* models of *Aif* loss have not been generated owing to its apparent function in early embryonic development²¹. In *Hq* mutant cerebellar granule cells, the loss of AIF leads to increased hydrogen peroxide sensitivity that can be rescued by AIF transduction. The role of AIF as a hydrogen peroxide scavenger is further supported by the increased peroxide-resistance of wild-type granule cells overexpressing AIF. *In vivo*, knockdown of AIF expression leads to oxidative stress and ultimately to neuronal degeneration. Therefore, we suggest that the AIF protein carries out two important functions in the cell. Under normal physiological conditions, AIF resides in the mitochondrial membrane to act as a free radical scavenger, specifically acting on hydrogen peroxide. Under conditions leading to apoptosis, AIF translocates from the mitochondria, resulting in large-scale DNA fragmentation.

Similar to many gene products involved in neurodegenerative and other disorders, the expression pattern of AIF is much wider than the tissues phenotypically affected by its downregulation³⁸. In addition to cerebellar neurons, AIF is expressed in the hippocampus, dentate gyrus, olfactory bulb, cerebral cortex and various brainstem nuclei (data not shown). However, in *Hq* mutant mice aged up to 26 months, neuron loss was not generally observed in non-cerebellar brain structures, although occasional mice had a thinning of the ventral surface of the dentate gyrus (data not shown). Our data and the data from others^{31,39} suggest that different neuronal types have varying endogenous sensitivity to peroxide damage. Resistant neurons may have additional peroxide scavenging systems or other mechanisms to cope with peroxide production and therefore are less sensitive to downregulation of AIF.

The *Hq* mutant mouse provides the first *in vivo* model for studying the role of oxidative stress on aberrant cell cycle re-entry and subsequent apoptosis. This unscheduled cell cycle re-entry may be mediated by abnormal gene expression, including that of mitogens, caused by oxidative stress, or more specifically, hydrogen peroxide. Hydrogen peroxide has been implicated in triggering the expression of mitogen-activated protein (MAP) kinases, epidermal growth factor receptor and the serine-threonine kinase Akt (reviewed in refs 27, 40). Activation of MAP kinases has been suggested to be necessary for initiation of cyclin D1 expression and cell cycle re-entry of quiescent G0 fibroblasts (reviewed in ref. 41). Therefore, the increase of hydrogen peroxide in the *Hq* mutant mouse, as evidenced by the increases in catalase activity and total glutathione, could stimulate mitogenic signals leading to abortive cell cycle re-entry of terminally differentiated neurons. However, neurons that re-enter the cell cycle cannot proliferate and thus undergo apoptosis^{13,14,42}. Definition of the oxidative stress-induced

mediators underlying aberrant cell cycle re-entry will greatly enhance the identification of therapeutic strategies for neurodegenerative disorders. □

Methods

Genetic mapping

Hq arose on a CF1 outbred stock and was transferred to a B6CBACa-A^{w-1}/A (B6CBA) background. High-resolution genetic mapping of the *Hq* mutation was performed by typing N2 progeny of a (B6CBACa-A^{w-1}/A – *Hq*/Hq × CAST/Ei) × B6CBACa-A^{w-1}/A – *Hq*/Y backcross with simple sequence length polymorphism (SSLP) markers (MIT/Whitehead Center for Genomics) on the X chromosome.

Immunohistochemistry and electron microscopy

TUNEL assays were performed using the *in situ* cell death detection kit (Roche Diagnostics) following the manufacturer's protocol. Immunohistochemistry was performed as described previously using antibodies to calbindin-D28 (Swant, 1:1,500) and colorimetric detection with DAB⁴³. For immunofluorescence studies, processed paraffin sections were incubated with mouse monoclonal antibodies to: BrdU (1:50; Dako), Pcnα (1:50; Santa Cruz Biotechnology), Cdc47 (1:50; NeoMarkers), 8-OHdG (1:1,000; QED Bioscience); and rabbit polyclonal antibodies to: caspase 3 (1:50; NeoMarkers), activated caspase 3 (1:50; Cell Signaling), and Gfap (1:50; Dako). Rabbit polyclonal GABA_A receptor α6 (1:50; Chemicon) was used on cryosections of Z-fixed (Anatech Ltd) tissues. Expression was visualized with Cy3- or fluorescein isothiocyanate (FITC)-labelled donkey or goat secondary antibodies, or mouse IgG1 or IgG2a subtype-specific goat secondary antibodies. Electron microscopic studies were performed on osmium tetroxide/lead citrate-stained ultrathin sections as reported previously⁴⁴.

PCR analysis

Intron 1 of the *Aif* gene was isolated from a bacterial artificial chromosome (BAC) clone (RP23-305N1) using specific primers to exons 1 and 2 with the Expand Long Template PCR system (Roche Diagnostics), as per the manufacturer's protocol, and sequenced. We performed PCR analysis on genomic DNA from *Hq* mutant mice, B6CBA littermate controls, and CF1 mice using forward and reverse primers from intron 1 of *Aif* (5'-AGTGTCCAGTCAAAGTACCGGG-3' and 5'-CTATGCCCTTCTCCATGTAGTT-3', respectively) and a viral primer from the U3 region of the LTR from murine C-type ecotropic virus (Genbank accession number U63133; 5'-CCAGAACTGTCTCAAGGTTCC-3'). The splice junction of the ecotropic viral insertion was determined by RT-PCR analysis from random-primed total RNA of *Hq* mutant mice, and B6CBA littermate controls were performed using a forward primer from exon 1 of *Aif* (5'-CAGAGGCCGAAACAGAGGAA-3') and a reverse primer from the murine C-type ecotropic proviral envelope (5'-GGTGGTCAGTAGGACGGTGA-3'). Other strains genotyped for this insertion were: A/J, AKR/J, AU/SsJ, BALB/cByJ, BUB/BnJ, CAST/Ei, CBA/J, C3HeB/FeJ, C57BL/6J, C57BL/KsJ, CZECHII/Ei, DBA/2J, DA/HuSn, F/St, FVB/NJ, NOD/LtJ, NZB/BLN, NZW/LacJ, RIIS/J, SIM/Ut, SJL/J, SWR/J and WSB/Ei.

Western blotting

We prepared brain extracts as described previously⁴⁵. Blots were incubated with a goat anti-AIF (1:1,000, Santa Cruz), rabbit anti-Crm1 (1:1,000, Santa Cruz) or rabbit anti-human neuron-specific enolase (1:1,000, Scytek) before incubation with horseradish peroxidase-conjugated goat or rabbit secondary antibody, and developed with the ECL kit (Amersham).

Primary neuronal cultures

Hq mutant and wild-type granule cells and cortical neurons were obtained from postnatal day 7 (P7) and embryonic day 14.5 (E14.5) mice, respectively, as described previously⁴⁶. Cells received treatment on the seventh day in culture. Cell viability was determined using propidium iodide. Cells were counterstained with the nuclear stain Hoechst 33342. Experiments were performed in duplicate and entire fields of cells were counted until at least 300 (cortical neurons) or 1,000 (granule cells) cells had been counted. Statistical significance was determined by a one-tailed *t*-test with a Bonferroni correction.

The coding region of *Aif* was subcloned into the multiple cloning cassette of a GFP-expressing Moloney murine leukaemia virus backbone, upstream of the IRES-GFP³². The construct was transfected into the Phoenix packaging cell line (Orbigen Inc) as described previously⁴⁷. We collected virus-containing supernatants on the third day after transfection. Cerebellar granule cells from *Hq* and wild-type P7 mice were infected with retrovirus on day seven in culture by adding retrovirus-containing medium (1:4 dilution). After three days of infection, cells were treated with fresh medium containing hydrogen peroxide for 5 h, and cell viability was determined as described above. Experiments were performed in duplicate and at least 1,000 cells were counted. Statistical significance was determined by two-way analysis of variance (ANOVA) analysis.

Lipid peroxidation, catalase and glutathione assays

Lipid hydroperoxides were extracted from equivalent amounts of tissue following the manufacturer's procedure (Cayman Chemical). Proteins were extracted in western lysis buffer and quantified by Bradford assay (Sigma). Catalase activity was determined using the Amplex Red Catalase Assay Kit as per the manufacturer's protocol (Molecular Probes). Equivalent extract volumes to those used in the catalase assay were deproteinized using metaphosphoric acid (Aldrich) before performing the glutathione assay as per the manufacturer's protocol (Cayman). For all assays, statistical significance was determined by a *t*-test.

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Orbital forcing of the martian polar layered deposits

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Since the first images of polar regions on Mars revealed alternating bright and dark layers, there has been speculation that their formation might be tied to the planet's orbital climate forcing^{1–4}. But uncertainties in the deposition timescale exceed two orders of magnitude: estimates based on assumptions of dust deposition, ice formation and sublimation, and their variations with orbital forcing suggest a deposition rate of 10^{-3} to 10^{-2} cm yr⁻¹ (refs 5, 6), whereas estimates based on cratering rate result in values as high as 0.1 to 0.2 cm yr⁻¹ (ref. 7). Here we use a combination of high-resolution images of the polar layered terrains⁸, high-resolution topography⁹ and revised calculations of the orbital and rotational parameters of Mars to show that a correlation exists between ice-layer radiance as a function of depth (obtained from photometric data of the images of the layered terrains) and the insolation variations in summer at the martian north pole, similar to what has been shown for palaeoclimate studies of the Earth^{10–12}. For the best fit between the radiance profile and the simulated insolation parameters, we obtain an average deposition rate of 0.05 cm yr⁻¹ for the top 250 m of deposits on the ice cap of the north pole of Mars.

Of the Mars Orbiter camera (MOC) images acquired of both the north and south polar regions, only a fraction clearly resolve layering, owing to the limited exposure of layers in troughs, seasonal frosts, image quality or illumination conditions. We focus on one particular image (M00-02100; see Fig. 1) from the north polar cap because of its high quality and the length of section exposed. Although layers are exposed in the southern cap, the length of any section is too short to be useful. The layers evident in image M00-02100 are representative of one long trough in the northern cap located near 279° W 86° N, from which several other MOC images were acquired; these layers show a consistent stratigraphy over a strike of more than 100 km.

The actual composition of the polar layers is poorly known. The residual north polar cap consists largely of water ice, although a 1–2-m-thick layer of CO₂ ice is formed and removed annually¹³. The darkening agent is thought to be dust or low-albedo sands and the general interpretation is that the layers are formed of frozen volatiles (mainly water) and dust deposited by atmospheric suspension (for example, see ref. 3).

The reflected intensity varies with local slope, dust and water/ice proportions, and season (owing to CO₂ frost deposition in winter, and differential retention of CO₂ and H₂O ice in summer). Images M00-02100 and E03-02206 (one martian year later), both acquired in midsummer from the same trough, show an identical pattern of light and dark bands. The early springtime image M18-00804 does not show intensity reversals relative to the summer images, which suggests the absence of stair steps and of significant short-scale slope effects on the summer albedo profile¹⁴. Regardless of the actual origin of the intensity contrasts that define the layers in the images (textural, composition, illumination), they display regular patterns of reflectance with depth that can be used to test the orbital forcing theory.

Profiles of radiance were extracted orthogonal to the layers from image M00-02100, which was first straightened and then averaged along strike (Fig. 1). Concurrently acquired Mars Orbiter laser altimeter (MOLA) topographic data were used to convert the

profiles of radiance as a function of pixel to radiance as a function of depth (Fig. 1). A clear feature in the first half of this profile is the presence of three nearly identical cycles (N1, N2 and N3 in Fig. 1), on top of which are some higher-frequency variations.

The age of the north polar layered terrain is currently poorly constrained. Viking observations suggested a net 0.02 to 0.08 cm annual water loss from the north cap¹⁵, but recent analysis suggests the cap is near equilibrium¹⁶. The lower elevation of the north cap predicts a long-term average transport and accumulation of water at the north pole with respect to the south pole^{7,16,17}. The present retention of CO₂ frost on the south cap alters this relationship, but this may not represent the average behaviour over the last million years¹⁷. On the basis of crude estimates of dust deposition rates, accumulation rates ranging from about 10^{-3} to 10^{-2} cm yr⁻¹ have been proposed^{5,6,18}. In contrast, the lack of any impact craters over 300 m in diameter led Herkenhoff and Plaut⁷ to estimate the age of the surface at less than 120,000 yr, corresponding to a resurfacing rate of 0.1 to 0.2 cm yr⁻¹. As the total thickness of section we analyse

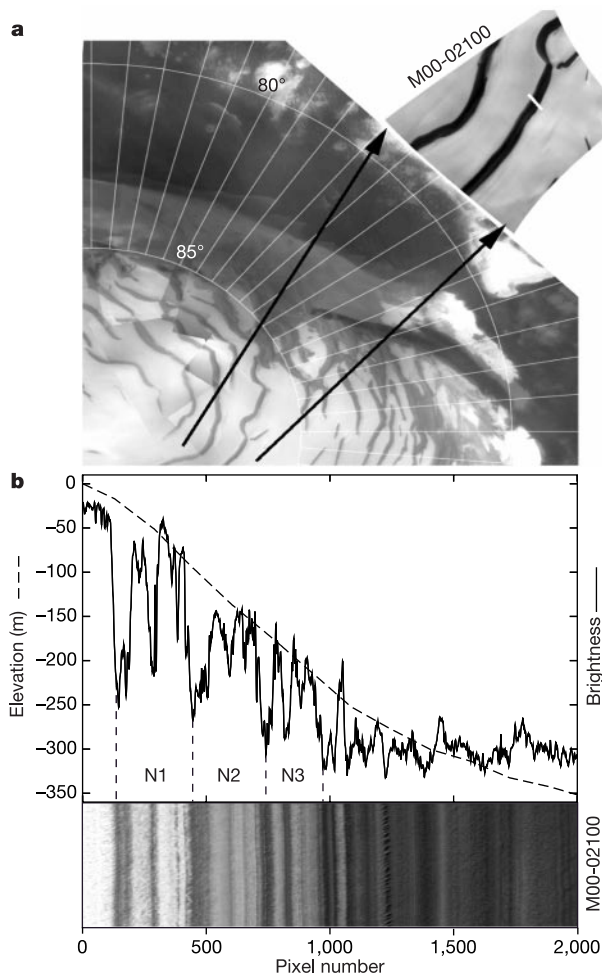


Figure 1 North pole layered terrains. **a**, Regional mosaic of Mars Orbiter camera (MOC) wide-angle camera images of the martian north pole showing the location of MOC narrow-angle image M00-02100. Circles of latitude 80° and 85° are plotted. **b**, The brightness profile (solid curve) versus pixel number for a section of the MOC narrow-angle image M00-02100 (bottom), is obtained by averaging the pixel value along vertical lines. This picture was taken by the MOC on 13 April 1999 (solar longitude $L_s \approx 123^\circ$), at latitude 86.48°. The original image was processed in order to straighten it. The dashed line is the elevation resulting from the Mars Orbiter laser altimeter (MOLA) experiment measurements (in metres on the y axis, with origin at pixel 0). MOLA data were used to convert the profile from horizontal distance along the x axis to depth. There are 13 MOLA spots across the image, and the elevation is interpolated between the topographic measurements. We observe three similar cycles (N1, N2, N3) between pixels 100 and 1,000.

is about 350 m, we can estimate from published deposition rates that the formation time for our section ranges from 100,000 to 35,000,000 yr.

Cutts and Lewis⁴ proposed hypothetical stratigraphic sequences using climatic models of deposition, but comparison with measurements of layers was not possible with Viking Orbiter images resolution⁴, about 30–50 times lower than the MOC (with up to 1.5 m pixel^{-1}). Here we directly compare the brightness profile of Fig. 2 to the north pole summer insolation in order to constrain the possible age of the deposit.

The changes in insolation at the surface of Mars arise from the variation of the planet's orbit resulting from the secular perturbations of all the other planets in the Solar System (see for example,

ref. 19), and from the precession and obliquity variations of the spin axis of the planet. The orbital motion is obtained through a new numerical integration of the whole Solar System, including all nine main planets, the Moon as a separate object, Earth and solar oblateness, tidal dissipation in the Earth–Moon system and the effect of general relativity. This new solution was also adjusted and compared to the JPL ephemeris DE406 (ref. 20). For Mars, the maximum discrepancy in longitude over 3,000 yr (the range of DE406) is less than 0.2 arcsec, and 3×10^{-8} in eccentricity. The obliquity and precession of the Mars axis were computed using initial conditions deduced from the Pathfinder mission²¹. The most striking feature of the solution (Fig. 2) is that at about 5 Myr, the obliquity increases, reaching more than 45° which induces a large increase of the summer insolation in the north pole. The main uncertainty in the obliquity solution arises from the initial precession frequency ($p = -7.576 \pm 0.035 \text{ arcsec yr}^{-1}$ (ref. 21)). We have verified that even when doubling this uncertainty ($p = -7.576 \pm 0.070 \text{ arcsec yr}^{-1}$) all solutions within this range lead to a similar increase of obliquity after 5 Myr, and behave as in Fig. 2d over 10 Myr, the chaotic behaviour of the solution^{19,22,23} being only significant beyond 10 Myr.

To compare the photometric profile with insolation, the trend of radiance that appears to be strongly correlated with depth (Fig. 1) is removed and the data are scaled, resulting in the radiance profile shown in Fig. 3. We assume that the top of the section has an age of 0, although possible deviations of a few thousand years should not significantly affect our results.

The shortest possible timescale present in the insolation curve is related to the climatic precession with a period of about 51,000 yr, while the main period in obliquity is of about 120,000 yr, and 95,000 to 99,000 yr in eccentricity, with a strong modulation of period 2.4 Myr owing to the near resonance of the secular proper modes g_3

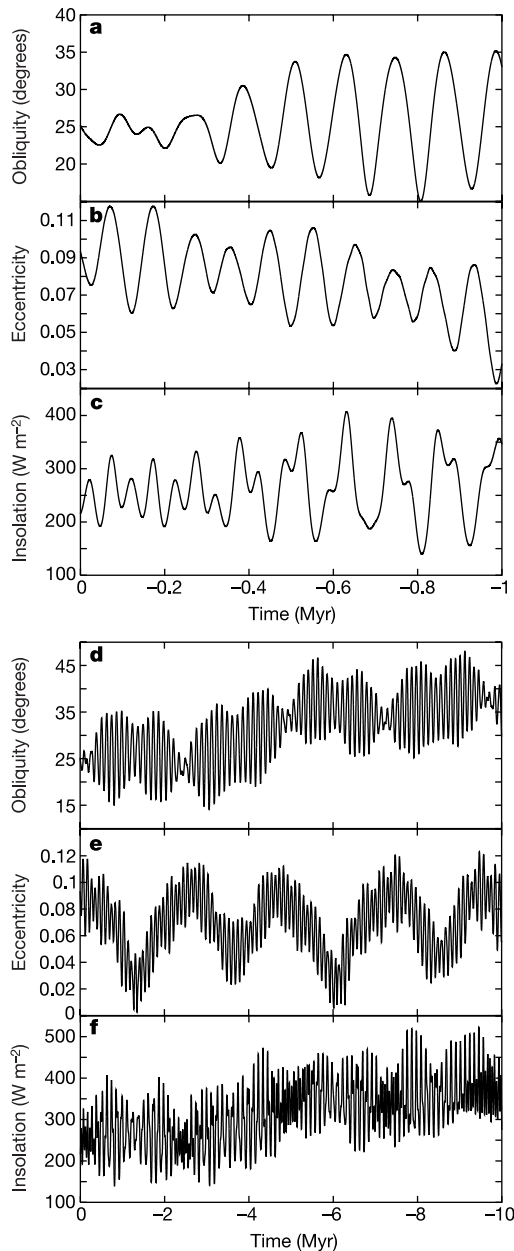


Figure 2 Obliquity, eccentricity and insolation at the north pole surface at the summer equinox ($L_S = 90^\circ$). **a–c**, Over 1 Myr; **d–f**, over 10 Myr. The orbital data are the result of a numerical integration of the whole Solar System, including the Moon, Pluto, tidal dissipation terms, solar and Earth oblateness. In the first 0.5 Myr of **c**, as the obliquity variations are small, the insolation is dominated by precession, while in the remaining part, the insolation variations becomes dominated by the obliquity cycle. (These data are available by request to J.L.)

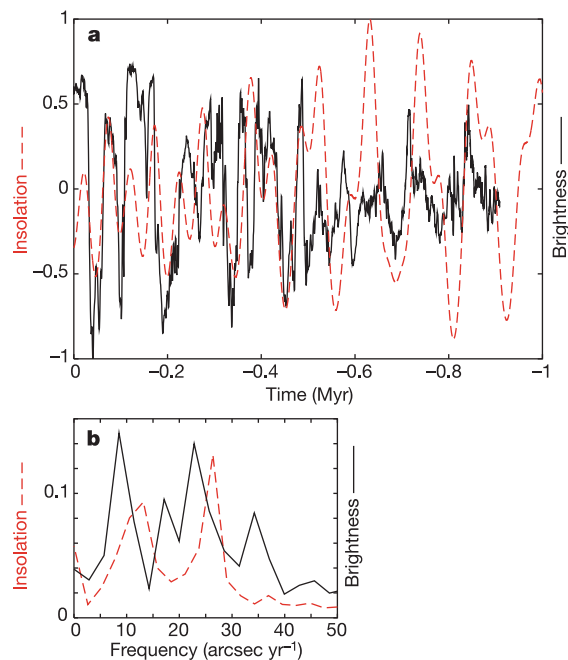


Figure 3 Comparison of brightness profile with insolation. **a**, Best fit of the brightness pattern from Fig. 1 (solid black curve) to the summer insolation at the north pole (Fig. 2c) (dotted red curve), after a transformation from pixel data to depth through the MOLA altimetry curve (Fig. 1) and, for the data beyond pixel 1,000, a division by 2 of the deposition rate. **b**, Spectral analysis of the insolation (dotted red) and the brightness pattern (solid black) over 0.5 Myr. Two main peaks are identified in both data. Owing to the short time span, in the insolation spectrum, the eccentricity and obliquity main frequencies are merged into a single line around $10 \text{ arcsec yr}^{-1}$. The second peak of the insolation curve (red) corresponds to climatic precession frequency ($25.4 \text{ arcsec yr}^{-1}$).

and g_4 (refs 19, 24).

Owing to the weak constraint imposed by the deposition models, a large variety of timescales are possible, but we are able to rule some of them out. For example, if we try to associate the 2.4-Myr eccentricity period (Fig. 2e) to the main cycles of Fig. 1, the third cycle (from pixel 700 to 1,000) will span from 6 Myr to 8 Myr. But in this range, the obliquity, and thus the summer insolation, becomes very high (Fig. 2d, f). For obliquity of about 45° , the sublimation rate becomes so large ($\approx 10 \text{ cm yr}^{-1}$) that the whole ice cap may be evaporated in about 10,000 yr (ref. 25), unless some dark residual deposit acts as a barrier for this evaporation². In any case, it is thus doubtful that the response pattern of the ice deposit will be the same during the first low obliquity cycle and this high obliquity cycle. We therefore discarded this possibility, and preferred to consider that the shortest significant period in the pattern corresponds to the precession cycle, as it appears in the insolation curve in Fig. 2c. In this case, we recover a very good agreement between the first three photometric cycles and the three insolation cycles up to about 500,000 yr. In addition, the spectral analysis of the first 0.5 Myr of the insolation curve and the data (Fig. 3) reveal in both cases two main peaks at very close locations, supporting our analysis.

Although we considered only the average deposition rate for the transfer function from depth to time, short scale variations of this deposition rate should not significantly affect our results. Moreover, a significant change of deposition rate with insolation^{2,17,25} would not change the main frequencies of the response, but only add additional harmonics, which could very well be present. Major erosional episodes may have caused unconformities and discontinuities in the layer accumulation³, but on the basis of the regular layer expression and periodic patterns in the N_1 , N_2 and N_3 cycles, we assumed that such large unconformities are not present in this first part of the record.

After 0.5 Myr, the amplitude of the obliquity (and thus also insolation) variations increase significantly. We assumed that the sublimation of the ice was larger during these periods, and thus that the effective deposition rate was smaller. In the same way, the decrease in atmospheric pressure at small obliquities will decrease the frequency and intensity of dust storms². We expressed this hypothesis by dividing by an empirical factor of 2 the deposition rate in our transfer function from MOLA topography to time.

The photometric curve is then directly compared with the insolation curve in Fig. 3, with a very satisfactory agreement, compared to usual Earth palaeoclimate records^{10–12}. With this process, we find that the time span for the whole 350 m layer is 900,000 yr. The first three main cycles, spanning 250 m, are accumulated in 500,000 yr, which corresponds to a deposition rate of 0.05 cm yr^{-1} , whereas in the remaining 400,000 yr, this value decreases to about 0.025 cm yr^{-1} .

The conjunction of high radiance level with high insolation value in summer fitted slightly better than the opposite (which would not change the timescale, but induces a shift of about 25,000 yr on the whole set). In fact, the deposition mechanism is not well known²⁶, and both hypotheses are possible. In the former, which we prefer, during the last precession-dominated 0.5 Myr (Fig. 3), the northern winter deposits are darkened by the most intense dust storm activity resulting from high summer insolation in the southern hemisphere (that is, low summer insolation in the northern hemisphere)^{27,28}. In the second scenario, the dark bands are the result of ice sublimation due to high insolation in summer at the north pole. When the obliquity forcing becomes dominant (after 0.5 Myr), the scenario may change, but we preferred here to focus on the first part of the record, where the signal is most apparent.

It is clear that additional data would be welcome to firmly establish this timescale for the north polar deposit, although the proposed timescale best fits the available data with minimal adjustments; however, we are very far from being able to core the martian ice caps as was successfully done on the Earth¹². As we had to slightly

modify the deposition rate during the second part of the record, we consider the timescale for the first part, where the photometric signal is the strongest, to be more reliable than that obtained in the second part. Finally, we stress again that the depositional/erosional mechanisms for the formation of the polar caps remain highly unconstrained. We also leave as an open question whether the observed 3-km-thick north polar cap²⁹ could have been formed during the recent 5-Myr period of low obliquity, which would correspond to an average deposition rate close to our 0.05 cm yr^{-1} value. □

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Artificial charge-modulation in atomic-scale perovskite titanate superlattices

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The nature and length scales of charge screening in complex oxides are fundamental to a wide range of systems, spanning ceramic voltage-dependent resistors (varistors), oxide tunnel junctions and charge ordering in mixed-valence compounds^{1–6}. There are wide variations in the degree of charge disproportionation, length scale, and orientation in the mixed-valence compounds: these have been the subject of intense theoretical study^{7–11}, but little is known about the microscopic electronic structure. Here we have fabricated an idealized structure to examine these issues by growing atomically abrupt layers of $\text{LaTi}^{3+}\text{O}_3$ embedded in $\text{SrTi}^{4+}\text{O}_3$. Using an atomic-scale electron beam, we have observed the spatial distribution of the extra electron on the titanium sites. This distribution results in metallic conductivity, even though the superlattice structure is based on two insulators. Despite the chemical abruptness of the interfaces, we find that a minimum thickness of five LaTiO_3 layers is required for the centre titanium site to recover bulk-like electronic properties. This represents a framework within which the short-length-scale electronic response can be probed and incorporated in thin-film oxide heterostructures.

In perovskites, charge ordering results in modulations of the electron density in the form of planes and slabs, whereas in lower-dimensional perovskite-derived systems, charge ordering leads to stripes, or one-dimensional charge modulations. Approximations to the first case can be realized in thin-film superlattices in which the formal valence of the transition-metal ion is varied. Superlattices of SrTiO_3 and LaTiO_3 are addressed here, where the titanium valence is varied from $4+$ to $3+$. SrTiO_3 is a band insulator with an empty d band, whereas LaTiO_3 has one d electron per site, and strong Coulomb repulsion results in a Mott–Hubbard insulator¹². Superlattices of these two perovskites capture many of the important aspects of naturally occurring charge-ordered systems, namely mixed-valence configurations near half-filling. The lattice constants are relatively well matched (for SrTiO_3 , $a_0 = 3.91 \text{ \AA}$; LaTiO_3 , pseudocubic $a_0 = 3.97 \text{ \AA}$), and the continuity of the TiO_6 octahedral lattice across the superlattice minimizes the perturbation of the electronic states near the chemical potential^{13,14}. The principal growth issue reduces to the control of the titanium oxidation state, which we have recently addressed for bulk-like film growth¹⁵.

We grew $\text{SrTiO}_3/\text{LaTiO}_3$ superlattice films in an ultrahigh-vacuum chamber (Pascal) by pulsed laser deposition, using a single-crystal SrTiO_3 target and a polycrystalline $\text{La}_2\text{Ti}_2\text{O}_7$ target. Extreme care was taken to start with atomically flat, TiO_2 -terminated SrTiO_3 substrates, which exhibited terraces several hundred nanometres wide, separated by $3.91\text{-\AA}$ unit cell steps as observed by atomic force microscopy¹⁶. A KrF excimer laser with a repetition rate of 4 Hz was used for ablation, with a laser fluence at the target surface of $\sim 3 \text{ J cm}^{-2}$. The films were grown at 750°C with an oxygen partial pressure of 10^{-5} torr , which represented the best compromise for stabilizing both valence states of titanium. Oscillations in the unit-cell reflection high-energy electron diffraction intensity were observed throughout the growth, and were used to calibrate the number of layers grown. After growth, the films were annealed in flowing oxygen at 400°C for 2–10 hours to fill residual oxygen vacancies.

Figure 1 shows the annular dark field (ADF) image of a superlattice sample obtained by scanning transmission electron microscopy (JEOL 2010F) of a 30-nm -thick cross-section along a substrate $[100]$ zone axis. In this imaging mode, the intensity of scattering scales with the atomic number Z as $Z^{1.7}$, so the brightest features are columns of La ions, the next brightest features are columns of Sr ions, and the Ti ions are weakly visible in between^{17–19}. The quality of the interfaces does not degrade with continued deposition, and the atomic step and terrace structure of the growing surface is maintained for hundreds of nanometres. The magnified view at the top of Fig. 1 shows a higher-resolution image, which visibly demonstrates the ability to grow a single layer of La ions. Because the layer is viewed in projection, roughness along the beam—particularly on length scales thinner than the sample—leads to apparent broadening. Thus these results represent an upper limit to the actual width of the layers.

With the same imaging conditions used to obtain Fig. 1, we analysed the energy of the transmitted electron beam and performed core level spectroscopy, atom column by atom column^{20–22}. This approach is able to probe internal structures directly, unlike surface-sensitive methods. Specifically, the titanium $\text{L}_{2,3}$, oxygen K, and lanthanum $\text{M}_{4,5}$ edges can be simultaneously recorded, with an energy resolution of $\sim 0.9 \text{ eV}$ and a spatial resolution slightly worse than the ADF resolution of $\sim 1.9 \text{ \AA}$, primarily owing to drift during the slower acquisition of the spectra. We obtained a scan through the Ti sites crossing a 2-unit-cell layer of LaTiO_3 (top centre panel of Fig. 2). By substituting La for Sr, there is locally an extra electron that resides mainly on the Ti d orbitals²³. To visualize this effect, the Ti $\text{L}_{2,3}$ near-edge structure can be decomposed into a linear combination of Ti^{3+} and Ti^{4+} , with no residual detectable above the experimental noise level (bottom panel of Fig. 2).

This decomposition, which would fail both conceptually and experimentally for more covalent materials, allows a particularly

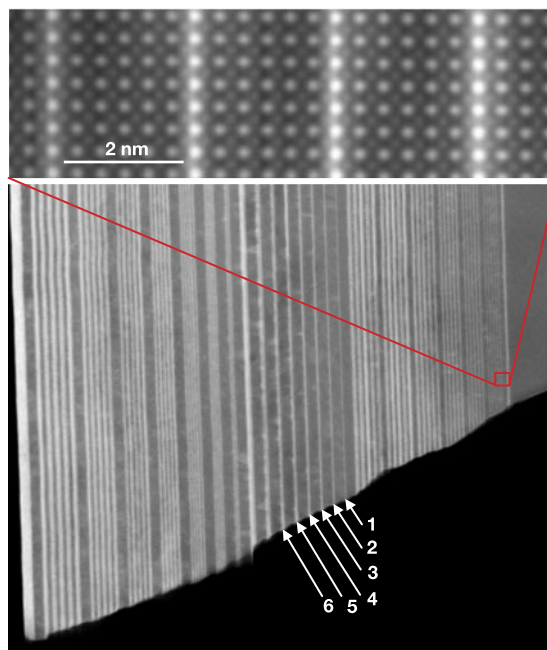


Figure 1 Annular dark field (ADF) image of LaTiO_3 layers (bright) of varying thickness spaced by SrTiO_3 layers. The view is down the $[100]$ zone axis of the SrTiO_3 substrate, which is on the right. After depositing initial calibration layers, the growth sequence is $5 \times n$ (that is, 5 layers of SrTiO_3 and n layers of LaTiO_3), $20 \times n$, $n \times n$, and finally a LaTiO_3 capping layer. The numbers in the image indicate the number of LaTiO_3 unit cells in each layer. Field of view, 400 nm . Top, a magnified view of the 5×1 series. The raw images have been convolved with a 0.05-nm -wide gaussian to reduce noise.

simple extraction of the distribution of the extra electron. The same trend has been observed qualitatively using Ti 2*p* X-ray absorption spectroscopy for bulk $\text{La}_{1-x}\text{Sr}_x\text{TiO}_3$, to which this technique is formally equivalent²⁴. This unusually ionic decomposition is probably the consequence of three effects: (1) the octahedral symmetry of the Ti sites is preserved; (2) the extra electron resides on the Ti site; and (3) the Ti 2*p*–3*d*–3*d* couplings in the presence of the core hole are large compared with the dispersion widths of the 3*d* bands²⁴. The onset of Ti^{3+} admixture, visible in the raw spectra, occurs in the vicinity of the double layer of LaTiO_3 , which is signalled by the La M_5 edge. The oxygen concentration across the layer remains unchanged within experimental error.

We also show the result of a similar scan across a single layer of LaTiO_3 (Fig. 3a). The spread of the extra electron can be seen in the fractional Ti^{3+} signal, which has a full-width at half-maximum of 19 ± 2 Å. Because this is significantly wider than the integrated La $M_{4,5}$ edge, the extended distribution of the extra electron can be considered intrinsic to the ideal structure, not limited by interface roughness or disorder. Figure 3b shows that the Ti^{3+} signal for both the single- and double-layer LaTiO_3 structures can be fitted with an exponential 'tail', giving a decay length of $\lambda = 1.0 \pm 0.2$ nm. As an

order-of-magnitude estimate of the expected screening length, we consider the Thomas–Fermi approximation for a degenerate semiconductor, assuming unity dopant activation. For the range of reported values for bulk and thin film SrTiO_3 (ϵ_0/m^* varying from 10 to 100, where ϵ_0 is the static dielectric constant and m^* is the effective mass), a screening length in the range 0.23–0.72 nm is deduced. This approximate correspondence with the experimental result is remarkable, given the borderline applicability of the effective mass approximation, the atomically abrupt potential variation, and the lack of consideration of the phonon structure of $\epsilon(q)$, the momentum-dependent dielectric response.

In Fig. 4a we compare the number of LaTiO_3 unit cells in a layer with the integrated Ti^{3+} area across the layer—the expected linear relationship may be seen. Note that the measured area is calibrated in an absolute sense, with the Ti^{3+} area observed per LaTiO_3 layer almost completely ($95 \pm 3\%$) accounting for the extra electron. More importantly, no evidence for compensating cation defects was observed. Figure 4b displays the peak fraction of Ti^{3+} as a function of the number of LaTiO_3 unit cells. An extrapolation of the existing data shows that 5 unit cells are needed for the central Ti site to exhibit bulk-like spectroscopic features.

In much of the region associated with the LaTiO_3 layers, the Ti sites exhibit mixed valence between 3+ and 4+. In bulk solid solutions of $\text{La}_{1-x}\text{Sr}_x\text{TiO}_3$, this configuration of the Ti valence results in metallic behaviour, and correspondingly, superlattices of $\text{LaTiO}_3/\text{SrTiO}_3$ are metallic. The overall conductivity is a function of the spacing between, and the thickness of, the LaTiO_3 interlayers.

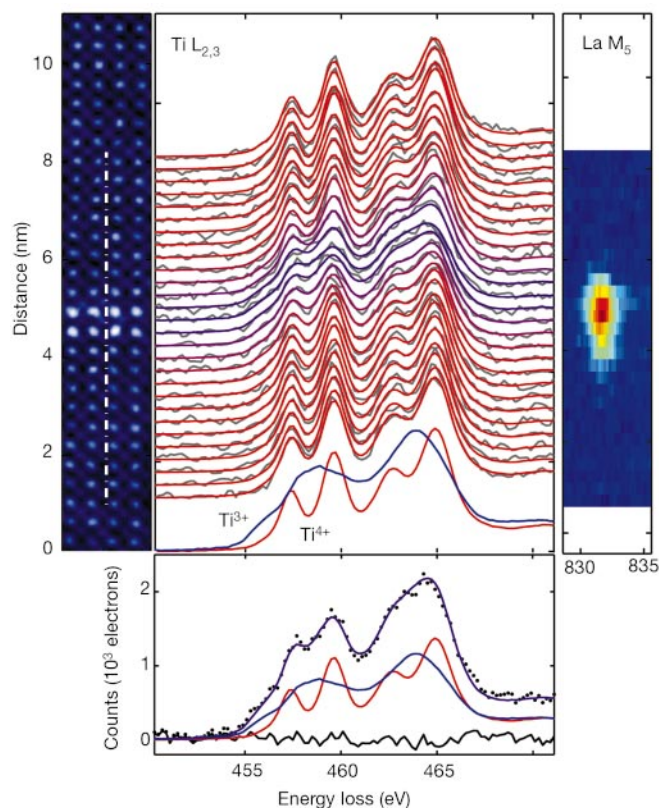


Figure 2 Electron energy-loss spectra (EELS) for La and Ti simultaneously recorded across a 2-unit-cell LaTiO_3 layer in SrTiO_3 . Left, the ADF image for the layer (layer '2' in Fig. 1); centre, the experimental Ti $L_{2,3}$ edge (grey lines), with the left side of the spectra aligned to the ADF image. The electron beam position for these data is denoted by the dashed line in the ADF image. Reference spectra for Ti^{4+} (red) and Ti^{3+} (blue) are shown at the bottom, taken from thick sections of SrTiO_3 and LaTiO_3 . Coloured lines show fits to the position-dependent spectra colour-coded by the fractional contribution of Ti^{4+} and Ti^{3+} . Right, the La M_5 edge, aligned to the ADF image. Note that scanning along the Ti sites broadens the La signal beyond the intrinsic resolution function. Bottom panel, detailed view of the decomposition of the Ti $L_{2,3}$ edge for the Ti site in the middle of the layer. Experimental data are shown as black dots, fitted (violet) by the addition of the reference spectra shown in red (Ti^{4+}) and blue (Ti^{3+}). The residual to the fit is given by the black line at the bottom.

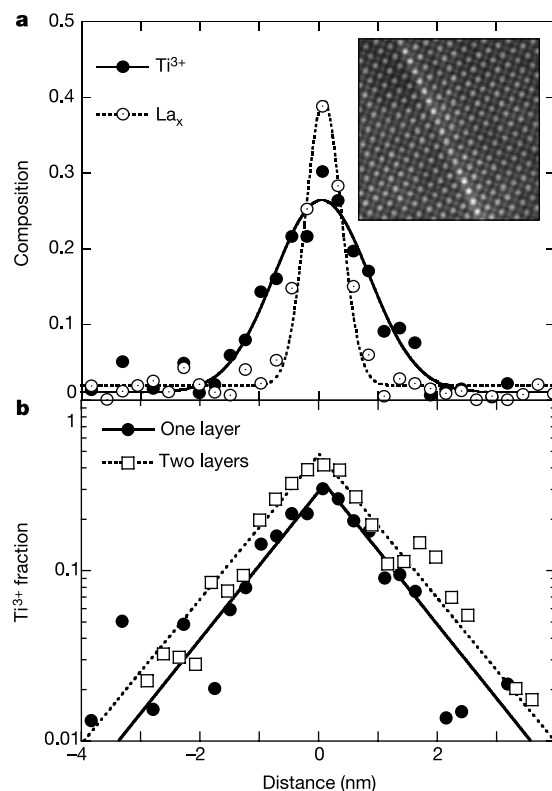


Figure 3 Spatial distribution of the Ti^{3+} signal in the vicinity of the LaTiO_3 layer and bilayer. **a**, EELS profiles for La and Ti recorded across a LaTiO_3 monolayer. Inset, the ADF image for the monolayer (layer '1' in Fig. 1). The La M edge is recorded simultaneously with the Ti L edge, yet the Ti^{3+} signal is considerably wider than that of the La. The absolute fractions of La and Ti^{3+} were calibrated from bulk LaTiO_3 and SrTiO_3 . **b**, The decay of the Ti^{3+} signal away from the LaTiO_3 monolayer of **a** as well as the bilayer of Fig. 2. The tails of the Ti^{3+} signal for both structures fit an exponential decay with a decay length of $\lambda = 1.0 \pm 0.2$ nm.

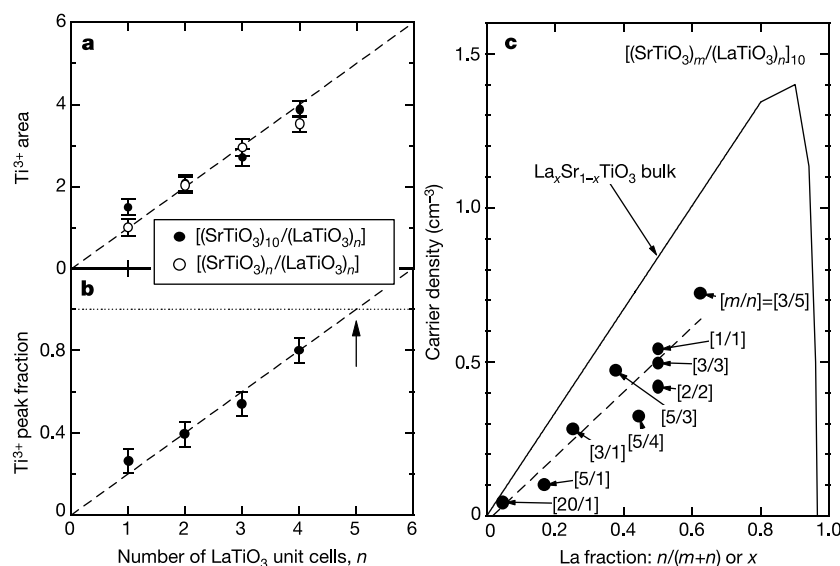


Figure 4 Summary of the electronic properties in SrTiO₃/LaTiO₃ superlattices as a function of the number of LaTiO₃ unit cells. **a**, The Ti³⁺ integrated area across LaTiO₃ layers 1–4 unit cells thick, as calibrated from bulk LaTiO₃. **b**, The Ti³⁺ peak fraction across these layers, extrapolating to 100% for 5-unit-cell-thick LaTiO₃. **c**, The carrier

density from Hall effect measurements for various superlattices of [(SrTiO₃)_{*m*}/(LaTiO₃)_{*n*}]₁₀, compared with bulk La_{*x*}Sr_{1-*x*}TiO₃ from refs 12 and 25. The dashed line represents 2/3 of the La contributing free carriers.

By examining the Hall effect in a number of superlattice compositions, we can measure the effective free carrier density *n*, which is nearly temperature independent ($n = -1/R_{\text{H}}e$, where R_{H} is the Hall coefficient and *e* is the electron charge). For a wide range of compositions, the measured carrier density corresponds to roughly 2/3 of the carriers expected from the La fraction (Fig. 4c).

In the artificial charge-modulated structures described here, the measured electronic structure reflects the static equilibrium distribution, including screening effects from the lattice. Although the charge modulation that we observe is driven by the presence of charged donor LaO layers, and not by spontaneous valence fractionation driven by strong correlation effects, the electronic response probed by this perturbation should be relevant for static charge ordering observed in bulk perovskites. Theoretical approaches addressing charge ordering range from those focusing on nearest-neighbour interactions in Hubbard and *t*-*J* models neglecting long-range Coulomb interactions, to those which require long-range interactions to stabilize inhomogeneous charge distributions^{7,8,10}. As a function of the relative strength of the local interactions, solutions vary from abrupt solitonic domain lines, to smoothly varying domain walls approaching conventional density wave structures⁹. The abrupt, short-length-scale features deduced from naturally occurring charge-ordered systems⁶ were not observed in our measurements. Indeed, in superlattice structures closer to half-filling, such as occasional SrTiO₃ layers within LaTiO₃, we see no short-length-scale structure, but rather a smooth average electron distribution (data not shown). Whether this is a consequence of the specific electronic parameters in the titanates, or a result of constraints arising from our artificial structure, remains to be understood. □

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Competing interests statement

The authors declare that they have no competing financial interests.

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Designing intermediate-range order in amorphous materials

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Amorphous materials are commonly understood to consist of random organizations of molecular-type structural units. However, it has long been known that structural organizations intermediate between discrete chemical bonds and periodic crystalline lattices are present even in liquids^{1,2}. Numerous models—including random networks and crystalline-type structures with networks composed of clusters^{3,4} and voids^{5,6}—have been proposed to account for this intermediate-range order⁷. Nevertheless, understanding and controlling structural features that determine intermediate-range order in amorphous materials remain fundamental, yet presently unresolved, issues^{7–9}. The most characteristic signature of such order is the first peak in the total structure factor, referred to as the first sharp diffraction peak or ‘low *Q*’ structure. These features correspond to large real-space distances in the materials, and understanding their origin is key to unravelling details of intermediate-range order. Here we employ principles of crystal engineering to design specific patterns of intermediate-range order within amorphous zinc-chloride networks. Using crystalline models, we demonstrate the impact of various structural features on diffraction at low values of *Q*. Such amorphous network engineering is anticipated to provide the structure/property relationships necessary to tailor specific optical, electronic and mechanical properties.

To generate specific void patterns in a host glass network as a means of understanding and designing complex patterns of intermediate-range order (IRO), we have exploited the crystal engineering strategies developed for the synthesis of metal-halide (MX) analogues of zeolites^{10–12}. Many of these halozeotype materials form glasses upon quenching a molten sample. The synthesis of these templated network glasses consists of a twofold strategy: (1) charge matching that allows network-modifying cations to template void structures within the host network without depolymerization of the MX₂ network; and (2) using alkylammonium cations that are the approximate size of an integral number of chlorine atoms (Fig. 1a) to provide a systematic templating motif with minimal disruption to the pseudo-close-packed anion sublattice of the ZnCl₂ network.

Other investigators have studied the effect of adding network-modifying AX salts to network glasses^{5,13,14}. However, for every templating [A]⁺ cation an additional [X][−] anion is added to the network. The additional anions reduce the need to form bridges between metals and result in network depolymerization. Network depolymerization is avoided in AgI-AgPO₃ glasses, for example, where anions of the modifying salt and the PO₃ network are chemically dissimilar, and thus do not disrupt the network with dangling bonds^{15,16}. However, in these silver phosphate glasses, considerable debate remains with regard to the distribution or clustering of the AgI within voids of the host network.

We have circumvented network depolymerization by a zeolite-type network modification whereby the addition of equimolar amounts of CuCl and the templating AgI salt of ZnCl₂ forms [Cu_{*n*}Zn_{*m*−*n*}Cl_{*2m*}]^{*n*−} templated networks; analogues to aluminosilicates, [Al_{*n*}Si_{*m*−*n*}O_{*2m*}]^{*n*−}. As in the network of ZnCl₂ itself, the chlorine atoms in these halozeotype-networks exhibit a pseudo-close-packed chlorine sublattice. However, in the network of CZX-1 (CZX = copper zinc halide) voids are generated in the MX₂ net-

work by trimethylammonium cations ([HTMA]⁺), which displace 4/16 of the cubic close-packed (c.c.p.) chlorine positions, and in α-CZX-4, methylammonium ([MA]⁺) or rubidium, cations displace 1/8 of the hexagonal close-packed (h.c.p.) chlorine positions. It is thus possible to envision that the structure of ZnCl₂-type networks are fundamentally defined by the approximate close packing of chlorine spheres in which zinc atoms fill a portion of the tetrahedral interstices. By choosing network-modifying templates that are equivalent in size to an integral number of chlorine atoms, and that bear a cationic charge sufficient to prevent template aggregation, we create recognizable structural modifications. Here we use the [MA]⁺, [HTMA]⁺ and [TPA]⁺ (tetrapropylammonium) cations, which are approximately equivalent in size to one, four and ten chlorine atoms, respectively (Fig. 1a). The concentration of the templating species, in addition to their varied sizes, can be manipulated to pattern specific arrangements of IRO in amorphous networks.

The neutron total structure factor was measured for a series of amorphous materials (Fig. 2) prepared using the perdeuterated alkylammonium cations, [d⁶-MA]⁺, [d¹⁰-HTMA]⁺, and [d²⁸-TPA]⁺, at AgI:CuCl:ZnCl₂ compositions of known or postulated crystalline models to probe the influence of the size and concentration of the templating cations on the IRO, as described in the Methods. Comparisons of the total structure factor of these templated glasses with that of amorphous zinc chloride^{17,18} demonstrates that the reciprocal space features $Q \geq 2 \text{ \AA}^{-1}$ ($Q = (4\pi \sin \theta)/\lambda = 2\pi/d$) exhibit only moderate variation between samples. (See also the comparative pair distribution function data in the Supplementary information Fig. 1 and Table 1.) Because of overlapping M–Cl and non-bonded D–Cl, D–C and D–N pair correlations it is not possible to extract reliable coordination numbers from the pair distribution function data. However, the general features of the *S*(*Q*) data and the atom–atom pair correlations are consistent with network architectures built from a common set of corner-shared MCl_{4/2} tetrahedra. By contrast, the

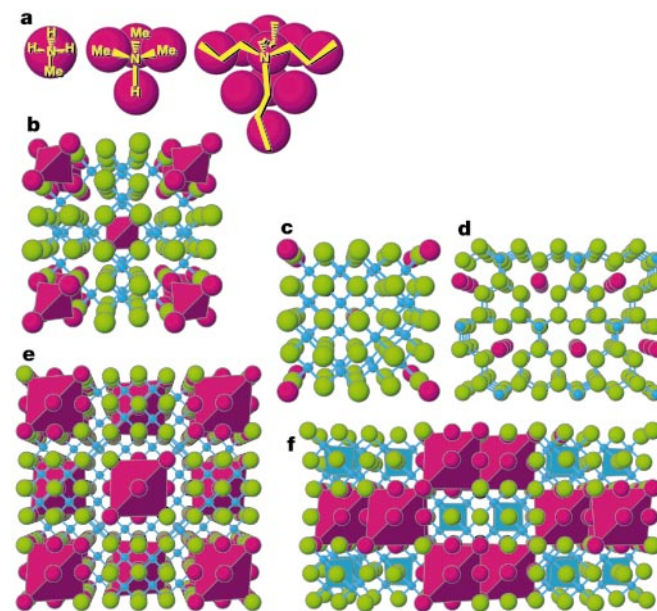


Figure 1 Structural models of templates and networks. Components of materials used. **a**, Comparison of alkylammonium templates to the close-packed assembly of one, four and ten chlorides. **b–f**, Network structures for **b**, CZX-1, [HTMA]₂Cu₂Zn₁₀Cl₂₄; **c**, CZX-5, [MA]₂M_{24-x}Cl₃₀; **d**, β-CZX-4, [MA]Cu₂Zn₂Cl₇; **e**, CZX-7, [TPA]₄M_{40-x}Cl₆₈; and **f**, CZX-6, [TPA]CuZn₃Cl₈ are given. The cation-filled network voids are represented by the purple-shaded polyhedra with purple spheres in sites where chloride ions have been displaced from the close-packed anion sublattice. Blue spheres correspond to metal sites and green spheres represent the chlorine atoms.

variations in the position and number of low- Q structures (LQS) in the structure factor data demonstrate that the presence of alkylammonium cations dramatically changes the IRO.

The experiments described here can be divided into two sets that separately probe the influence of the template size and network composition on the generation of IRO. (1) The network composition was held constant at $[A]-1:1:5$ ($ACl:CuCl:ZnCl_2$) while the size of the template was changed with $A = [TPA]^+$, $[HTMA]^+$ and $[MA]^+$, for the data reported in Fig. 2b, d and g, respectively. (2) The template/network compositions were varied while the size of the template was held constant for $[TPA]^+$ (Fig. 2b and c) and $[MA]^+$ (Fig. 2g and h).

The most pronounced LQS in Fig. 2b, d and g appear at higher values of Q as the size of the templating cation is decreased for materials of constant composition, demonstrating that the length scale of the IRO is directly related to the size of the templating cations. Furthermore, these LQS are distinct from the single LQS of $ZnCl_2$ (Fig. 2a). The large $[TPA]^+$ template gives rise to LQS of $0.65 \text{ \AA}^{-1}$, equivalent to that found for the $(AgI)_{0.5}(AgPO_3)_{0.5}$ and $(AgI)_{0.75}(Ag_2MoO_4)_{0.25}$ glasses; it is the lowest reported value for any network glass^{19,20}.

Although the origin of the LQS in the AgI modified glasses is

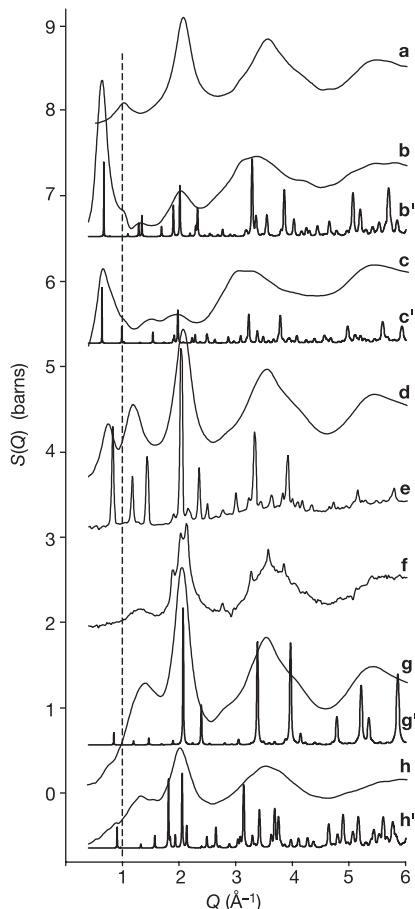


Figure 2 Total structure factors and power diffraction patterns for amorphous materials and crystalline models. Total structure factors: **a**, vitreous $ZnCl_2$; **b**, vitreous $[d^{28}\text{-TPA}]-1:1:5$; **c**, vitreous $[d^{28}\text{-TPA}]-1:1:3$; **d**, vitreous $[d^{10}\text{-HTMA}]-1:1:5$; **e**, devitrified and recrystallized $[d^{10}\text{-HTMA}]-1:1:5$, at 70°C ; **f**, recrystallizing vitreous $[d^6\text{-MA}]-1:1:5$ after 14 h; **g**, vitreous $[d^6\text{-MA}]-1:1:5$, 0 to 9 h after quenching (**h**) molten $[d^6\text{-MA}]-1:1:2$ at 275°C . Calculated power diffraction patterns for the model lattices: **b'**, CZX-6; **c'**, CZX-6; **g'**, CZX-5; and **h'**, β -CZX-4. The dashed line provides a comparison to the single low- Q structure of amorphous $ZnCl_2$. See the Supplementary Information for a plot of the pair distribution function data, a table of indexed Bragg-type lattice planes and a summary of the atom-atom pair correlations.

debated^{15,16}, we note that the commonly observed Ag_4I_4 molecular cluster, for example in $Ag_4I_4(PPh_3)_4$ (ref. 21), has a radius of about $4.8 \text{ \AA}$ compared with the radius of about $4.7 \text{ \AA}$ for the $[TPA]^+$ cation. We speculate that such Ag_4I_4 clusters, with exo-ligation to the glass network, provide the origin of similar LQS in AgI-modified oxide and $[TPA]^+$ -modified zinc chloride glasses.

The second notable feature in this series is the decrease in the relative intensity of the peak at $2 \text{ \AA}^{-1}$, which is the strongest peak in the $S(Q)$ of $ZnCl_2$ (ref. 18), as the size of the template increases. While the template/network composition was held constant, the larger templates require the relative amount of MCl_2 network per unit volume to decrease. With fewer network scatters per unit volume, the intensity of this scattering feature is decreased. A similar influence on the peak at $2 \text{ \AA}^{-1}$ is observed when the template/network composition is varied. The more template-rich compositions, $[TPA]-1:1:3$ (Fig. 2c) and $[MA]-1:2:2$ (Fig. 2h), exhibit a diminished peak intensity with respect to the $[A]-1:1:5$ glasses (Fig. 2b and g, respectively). These data are consistent with a lesser amount of the metal-chloride network per unit volume (that is, a greater volume fraction of template-generated network voids) in the template-rich materials. The variation in the template/network composition also shifts the position of several LQS that are indicative of variation in the template distribution.

The correspondence between the diffraction from the amorphous and crystalline materials provides insight into the origin of the IRO in these glasses. The $[HTMA]-1:1:5$ glass (Fig. 2d) devitrifies and recrystallizes to CZX-1 (Fig. 2e), the crystalline phase of the same composition. The similarity between the crystalline diffraction and the scattering of the glass suggests that consideration of the reciprocal lattice planes that give rise to the low- Q Bragg diffraction may provide insight into the nature of the IRO in the glass²². The pair distribution function of the glass and recrystallized samples (Supplementary Information Fig. 1d and e and Table 1) demonstrate that there is little change in the pair correlations, although there is a pronounced loss of resolution of the features at large distances in the glass.

The LQS at $0.75 \text{ \AA}^{-1}$, $1.20 \text{ \AA}^{-1}$, and the possible shoulder at about $1.4 \text{ \AA}^{-1}$, as well as the higher- Q features at $2.06 \text{ \AA}^{-1}$, and the broad peak at $3.5 \text{ \AA}^{-1}$ with a possible shoulder at about $4 \text{ \AA}^{-1}$, can be indexed as being related to reciprocal space reflections corresponding to a $10.6 \text{ \AA}$ body centred cubic (b.c.c.) unit cell (Fig. 1b and Supplementary Information Table 1). The $[HTMA]^+$ cations are distributed in a b.c.c. arrangement, each displacing $4/16$ of the close-packed chlorine sites to form a $[HTMA]_2[Cu_2Zn_{10}Cl_{24}]$ network. Using the crystalline structure of CZX-1 as a model from which to interpret the origin of the amorphous IRO, we note that the (222) plane at $2.05 \text{ \AA}^{-1}$ corresponds to stacking pseudo-close-packed layers of chlorine atoms. The observed d-spacing of $3.05 \text{ \AA}$ corresponds to approximately close-packed spheres with a Cl-Cl distance of $3.73 \text{ \AA}$. By contrast, the low- Q Bragg-type reflections indicate the position and orientation of the templating cations within the host network.

Using the scattering peak at $2 \text{ \AA}^{-1}$, corresponding to the approximately close-packed layers of chlorine atoms, that is common to all of these structure factor data as a point of reference, it is possible to index each network structure to a crystalline-type lattice. (Powder patterns calculated for the model structures are shown corresponding to each glass in Fig. 2 and observed and calculated numerical peak assignments are given in Supplementary information Table 1.)

For the $[MA]-1:1:5$ material, this peak at $2.05 \text{ \AA}^{-1}$ is indexed as the (222) Bragg-type reflection, with the primary LQS indexed as the (211) reflection, consistent with a $10.6 \text{ \AA}$ cubic unit cell with a b.c.c. arrangement of templates such as the model lattice $[MA]_2M_{24-x}Cl_{30}$ (CZX-5) ($M = Cu$ and/or Zn ; $x = 9$ for an ideal MX_2 -network) of Figs 1c and 2g. This lattice is similar to that of CZX-1, except that the smaller $[MA]^+$ displaces only $1/16$ of the anion sites. The splitting of the LQS peaks observed for the $[MA]-1:2:2$ melt

suggests a lowering of symmetry; and the three discernible LQS features can be indexed as the (100), (001) and (110) Bragg-type reflections of a hexagonal lattice with $a = 8.0 \text{ \AA}$ and $c = 6.5 \text{ \AA}$. This is consistent with the β -CZX-4 model (Figs 1d and 2h'), in which the required stoichiometry $[\text{MA}]\text{Cu}_2\text{Zn}_2\text{Cl}_7$ is achieved by placing a $[\text{MA}]^+$ cation on each corner of a hexagonal unit cell, replacing 1/8 of the chlorine sites. This liquid network polymorph is related to the orthorhombic crystalline α -CZX-4 (ref. 10) by shifting the columns of $[\text{MA}]$ -templated cages.

In the $[\text{TPA}]^+$ -templated materials the first LQS observed $Q = 0.65 \text{ \AA}^{-1}$ is 1/3 the value of the close-packed chlorine reference peak. For $[\text{TPA}]-1:1:5$ these index as (111) and (333) Bragg-type reflections, respectively, of a $16.2 \text{ \AA}$ cubic unit cell, which along with other observed reflections is consistent with the CZX-7 model structure, $[\text{TPA}]_4\text{M}_{40-x}\text{Cl}_{68}$ ($\text{M} = \text{Cu}$ and/or Zn ; $x = 6$ for an ideal MX_2 -network), with an f.c.c. distribution of $[\text{TPA}]$ cations (Figs 1e and 2b'). By contrast, the LQS observed for $[\text{TPA}]-1:1:3$ are better indexed to the tetragonal cell, with $a = 11.0 \text{ \AA}$ and $c = 22.0 \text{ \AA}$, corresponding to the CZX-6 model lattice, $[\text{TPA}]\text{CuZn}_3\text{Cl}_8$ (Figs 1f and 2c'). In this stuffed, non-interpenetrated ZnI_2 -type network, the $[\text{TPA}]^+$ templated voids share corners, and hence correspond to the size of only eight chlorine atoms, as opposed to the ten chlorine atoms for the isolated voids in the CZX-7 model network. In these $[\text{TPA}]^+$ -templated networks, the templates displace 10/27 and 1/2 of the close-packed chlorine sites in the CZX-7- and CZX-6-type networks, respectively, which accounts for the low intensity of the peak at $2 \text{ \AA}^{-1}$. Comparison of the compositional variants of the two $[\text{MA}]^+$ -templated and two $[\text{TPA}]^+$ -templated materials demonstrates that the length scale of the correlations giving rise to the IRO is inversely proportional to the template concentrations, owing to the larger template-to-template distances at higher dilution.

The concept of using zeolite-type charge-matching strategies to prevent network depolymerization, along with specifically tailored templates to design complex patterns of IRO in amorphous networks can readily be extended to numerous other classes of materials. Similar patterns of IRO can probably be engineered into amorphous networks of heavier halides (Br and I) and chalcogenides (S, Se and Te). Oxide and fluoride network glasses can similarly be templated using the zeolitic synthetic strategy, and the numerous known zeolite and silicate clathrate crystal structures can provide lattice models for interpreting the IRO. It is also likely that Zintl-type clusters could template similar patterns of IRO in amorphous alloys^{23–25}.

The use of crystal analogies to interpret the IRO does not imply that the glasses or liquids can be described as nanocrystallites, but rather that atomic-density fluctuations in the material exhibit characteristics analogous to crystalline unit cells⁶. Static models of infinite lattices need further modification to account for the disorder and dynamics that are fundamental to structure/property relationships in amorphous materials. Nevertheless, the IRO of network glasses provides a useful medium within which to organize functional templates on nanometre-length scales. □

Methods

Preparation of materials

The alkylammonium-templated copper zinc chloride materials were prepared by grinding appropriate molar mixtures of AgCl , CuCl and ZnCl_2 together in a mortar and pestle within a nitrogen-filled glove box. CuCl and ZnCl_2 starting materials were dried under vacuum at 250°C , then double-sublimed before use. (For ZnCl_2 this process yields exclusively the anhydrous δ -phase.) Deuterated amines or ammonium salts (99.99%) were purchased from Cambridge Isotopes, Isotec, and MSD Isotopes and dried before use. These materials were then placed within fused silica ampoules (8 mm outer diameter $\times$ 6 mm inner diameter) that were sealed and heated to 300°C . Glasses of these materials were formed by quenching the ampoule with the molten material into an ice bath. Typical reactions utilized 10 g of material such that more than 1.5 cm of a solid glass rod was obtained within the fused silica ampoule. Materials prepared at the 1:1:5 composition (the composition of crystalline CZX-1; ref. 11) were examined for each templating salt, as well as materials with the $[\text{MA}]-1:2:2$ composition of CZX-4 (ref. 10) and the $[\text{TPA}]-1:1:3$ composition predicted for CZX-6. The glasses formed with the $[\text{MA}]^+$ cations begin to devitrify and

crystallize at room temperature. After 12 to 14 h of data collection the growth of CZX-4 Bragg peaks was observed for $[\text{MA}]-1:1:5$ (Fig. 2f). The glass formed by quenching a melt of CZX-4, $[\text{MA}]-1:2:2$, persisted less than 1 h at room temperature, so the structure factor for this material was measured at 275°C (about 50° above the melting point). The glass formed from $[\text{HTMA}]^+$ does not devitrify and recrystallize until it is heated above about 60°C . No glass-to-crystal transition is observed upon heating the $[\text{TPA}]^+$ material; however, differential scanning calorimetry (DSC) studies indicate a glass transition temperature around 60°C .

Total structure factors

Total structure factor data were obtained on the GLAD diffractometer at the IPNS at Argonne National Laboratory. Data were collected on vitreous samples in the fused silica ampoules, described above, at room temperature (except for the melt of $[\text{MA}]-1:2:2$, at 275°C). Data were also collected for a vitreous sample of the ZnCl_2 starting material. These data were corrected with respect to an empty fused silica tube, a vanadium standard, and the instrument background. Structure factor and pair distribution functions were calculated using the suite of programs provided by the IPNS-GLAD facility. Removal of the LQS from the structure factor data before the Fourier analysis has virtually no effect on the pair distribution function, thus giving no additional hint as to the nature of the IRO²⁶.

Lattice constants

Lattice constants were calculated for model crystalline structures that are based on a pseudo-close-packed anionic sublattice, using the respective measured Cl–Cl pair correlations. However, for the $[\text{MA}]-1:2:2$ melt, the maximum in the Cl–Cl pair correlation at $3.8 \text{ \AA}$ is significantly broadened to larger distance. Using a larger average Cl–Cl distance of $3.99 \text{ \AA}$, a hexagonal cell of $8.0 \times 6.5 \text{ \AA}$ is proposed which provides an excellent match to the observed reciprocal space features.

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Competing interests statement

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Self-organization of supramolecular helical dendrimers into complex electronic materials

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The discovery of electrically conducting organic crystals¹ and polymers¹⁻⁴ has widened the range of potential optoelectronic materials⁵⁻⁹, provided these exhibit sufficiently high charge carrier mobilities⁶⁻¹⁰ and are easy to make and process. Organic single crystals have high charge carrier mobilities but are usually impractical¹¹, whereas polymers have good processability but low mobilities^{1,12}. Liquid crystals exhibit mobilities approaching those of single crystals and are suitable for applications¹³⁻¹⁸, but demanding fabrication and processing methods limit their use. Here we show that the self-assembly of fluorinated tapered dendrons can drive the formation of supramolecular liquid crystals with promising optoelectronic properties from a wide range of organic materials. We find that attaching conducting organic donor or acceptor groups to the apex of the dendrons leads to supramolecular nanometre-scale columns that contain in their cores π -stacks of donors, acceptors or donor-acceptor complexes exhibiting high charge carrier mobilities. When we use functionalized dendrons and amorphous polymers carrying compatible side groups, these co-assemble so that the polymer is incorporated in the centre of the columns through donor-acceptor interactions and exhibits enhanced charge carrier mobilities. We anticipate that this simple and versatile strategy for producing conductive π -stacks of aromatic groups, surrounded by helical dendrons, will lead to a new class of supramolecular materials suitable for electronic and optoelectronic applications.

We have elaborated a library based on a semifluorinated tapered dendron^{19,20} that was functionalized at its apex with a diversity of electroactive donor (D) and acceptor (A) groups, (3,4,5)12F8G1-D/A, (Figs 1 and 2). The resulting functional dendrons self-assemble into columns containing the optoelectronic element in their cores. For synthesis and structural analysis see the Supplementary Infor-

mation. The supramolecular columns self-organize into homeotropically aligned liquid crystals with hexagonal columnar (Φ_h), centred and simple rectangular columnar (Φ_{r-c} and Φ_{r-s}) morphologies (Fig. 2). We name this desirable homeotropic self-organization between electrodes 'self-processing'.

Carbazole (D1), naphthalene (D2) and pyrene (D3 and D4) derivatives have been introduced at the apex as D groups and 4,5,7-trinitrofluorenone-2-carboxylic acid (TNF) as an A group (A1) (Fig. 1). We use a diethylene glycol or tetraethylene glycol spacer between the dendron and the D or A groups to decouple their motion and facilitate fast self-assembly. The approach is simple and allows for diverse synthetic methods, and ensures that the self-assembled D and A groups are protected from moisture by the internal compartmentalization of the column and external jacketing with a fluorinated coat (Fig. 2).

Co-assembling a D-dendron with an A-dendron incorporates an electron donor-acceptor (EDA) complex in the centre of the column (see A1 + D1). Disordered amorphous polymers with A and D side groups form EDA complexes when the D-dendron (D1) is mixed with the A-polymer (AP1) and when the A-dendron (A1) is mixed with a D-polymer (DP1 or DP2). The EDA complexes assemble into columns, which in turn self-organize into Φ_h or Φ_r liquid crystals. The repulsion between the dendron's fluorine coat and the aromatic groups is so strong that the complexed polymer is forced to reside in the centre of the column (left and right columns in Fig. 2). EDA interactions²¹ rather than the previously reported covalent bonding²² are generating a supramolecular polymer²³ with dendritic side groups. But as in the previous case²², jacketing with dendritic side groups leads to a more ordered polymer backbone.

The self-assembly and self-organization of the material into Φ_h (D1, D2, D3, D4, D1 + A1, A1 + DP1, A1 + DP2), Φ_{r-s} (D1 + AP1) and Φ_{r-c} (A1) liquid crystals was demonstrated by a combination of techniques including differential scanning calorimetry (DSC), X-ray diffraction (XRD) (Fig. 3a), thermal optical polarized microscopy (TOPM) (Fig. 3b, c) and electron diffraction (ED) (Supplementary Information). Calculations based on density and XRD measurements indicate that between 3.8 and 4.6 dendrons self-assemble into a 4.8 Å stratum of the column (Supplementary Information). These self-processed systems have a column density of 4.5×10^{12} to 5.8×10^{12} columns per square centimetre (Supplementary Information), which exceeds the active element density achieved in other systems¹⁰ by two orders of magnitude.

Figure 1 summarizes the structures of the supramolecular assemblies and their electron mobilities (μ_e) and hole mobilities (μ_h). The field- and temperature-independent μ_e and μ_h values were determined by the time-of-flight (TOF) method (Supplementary Information). Tapered D-dendrons lead to μ_h ranging from 10^{-4} to 10^{-3} cm² V⁻¹ s⁻¹ in the liquid crystal state whereas the A-dendron (A1) has an μ_e of 10^{-3} cm² V⁻¹ s⁻¹. The mobilities μ of the EDA polymer complexes are in the same range. All these values are two to five orders of magnitude higher than those of the related D and A compounds in the amorphous state (Fig. 1), reflecting the π -stacked one-dimensional ordered structure of the D, A or EDA complexes in the centre of the supramolecular column. The charge migration through the columns is not well understood but may involve a polaron hopping process^{16,24}. XRD and ¹H-nuclear magnetic resonance (NMR) studies carried out in solution and bulk by using previously developed techniques²⁵ demonstrate π - π -interactions between the D or A groups. The μ value of these very simple D-dendrons are similar to those of more complex discotic liquid crystals¹³⁻¹⁶ produced from disc-like molecules; reports of discotic liquid crystals exhibiting higher μ values^{13,15,26,27} refer either to measurements on the Φ_{ho} crystal phase, or to measurements of the one-dimensional intracolumnar mobility by contactless pulseradiolysis time-resolved microwave conductivity (PR-TRMC)^{13,15,26,27}. TOF data are inherently lower owing to structural imperfections within the inter-electrode gap, but are more relevant

for technical applications than the higher values obtained by PR-TRMC.

Cooling the liquid crystal state leads to crystallization in the case of **D2**, whereas the two-dimensional (2D) order of **D1**, **D3** and **A1** is enhanced in the glassy state showing helical short-range order. For

example, the XRD of a fibre of **A1** in the glassy ordered state cooled from the liquid crystal phase exhibits, in the small-angle region, the pattern of the Φ_h order (Fig. 3a). This demonstrates that the liquid crystal order of the large and uniform homeotropic domain (100 μm to a few centimetres in size) obtained by slow cooling on

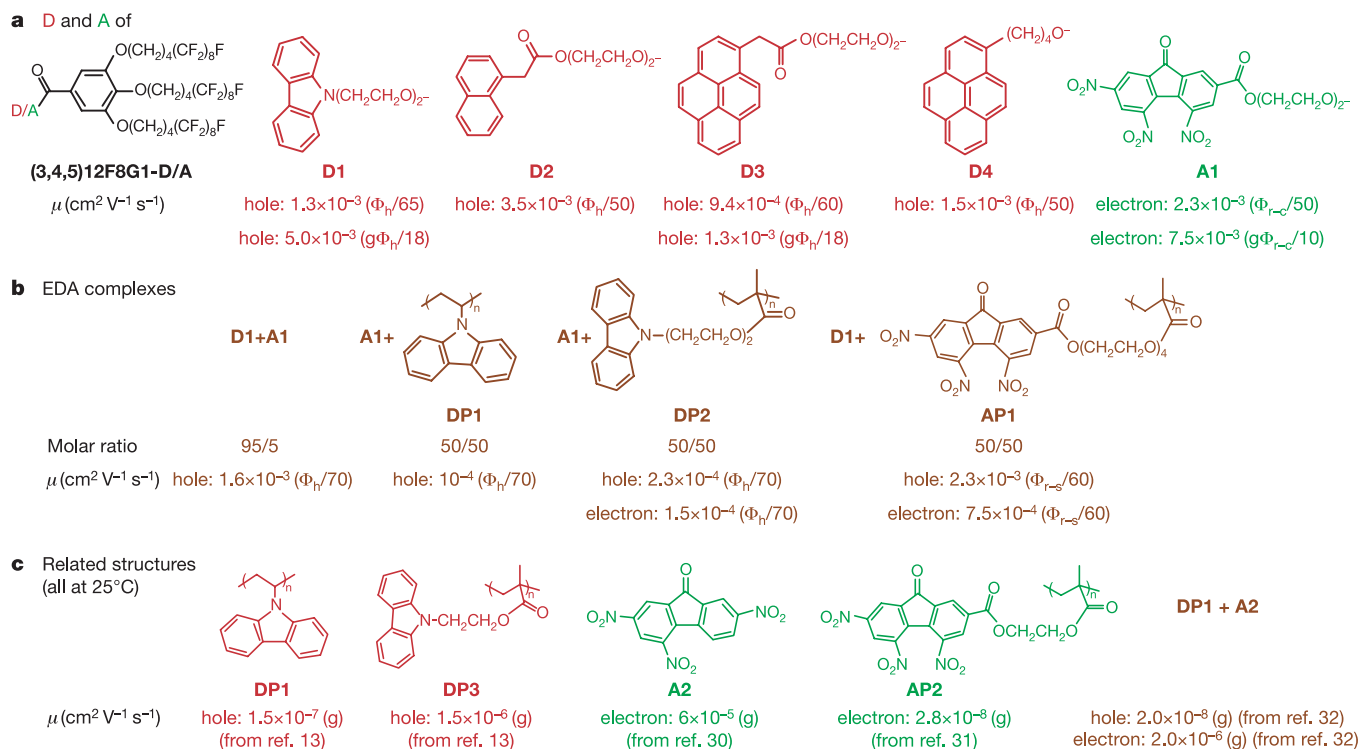


Figure 1 Charge carrier mobility of supramolecular columns and related systems.

a, Donor (**D1–4**) and acceptor (**A1**) groups of (3,4,5)12F8G1-D/A. **b**, EDA complexes. **P**, polymer. **c**, Related structures (all at 25 °C). The charge carrier mobility values (μ) are shown for all structures. ($\Phi_h/65$), in hexagonal columnar phase at 65 °C. ($\Phi_{r-c}/50$), in

centred rectangular phase at 50 °C. ($\Phi_h/18$), in glassy hexagonal columnar phase at 18 °C. ($\Phi_{r-c}/10$), in glassy centred rectangular phase at 10 °C. ($\Phi_{r-s}/60$), in simple rectangular phase at 60 °C. (g), glassy.

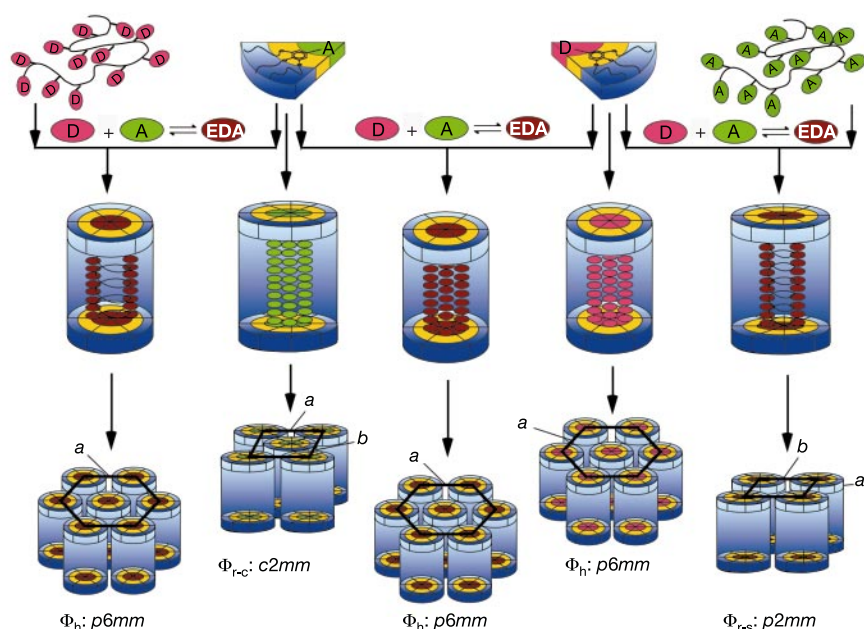


Figure 2 Schematic illustration of the liquid crystal assembly processes. Shown are the self-assembly, co-assembly and self-organization of dendrons containing donor (D) and acceptor (A) groups with each other and with disordered amorphous polymers containing

D and A side groups. The different systems form hexagonal columnar (Φ_h), centred rectangular columnar (Φ_{r-c}) and simple rectangular columnar (Φ_{r-s}) liquid crystals. a and b are lattice dimensions; space groups are shown.

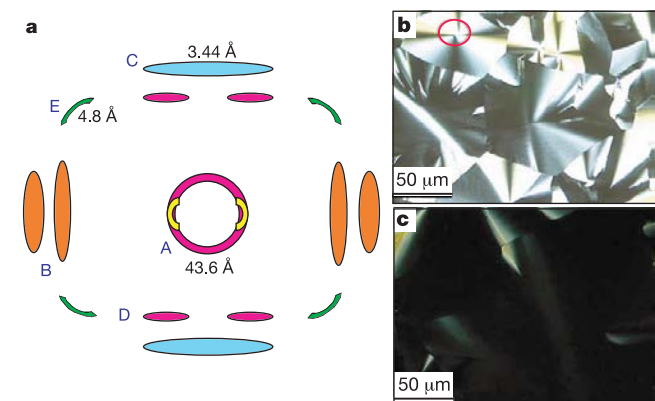


Figure 3 Structural characterization of self-assembled liquid crystals. **a**, Characteristic small- and wide-angle XRD of **A1**. The individual XRD features are identified as (A) supramolecular column to column distance, short-range, (B) order in tail region within a supramolecular cylinder, (C) π -stacks within the column, (D) correlation along a direction at an angle to the supramolecular column axis, (E) helical correlation. **b**, Thermal optical polarized micrograph (TOPM) of **D4** in the Φ_h liquid crystal state, obtained by cooling at $20^\circ\text{C min}^{-1}$ from the isotropic phase on a hydrophobic indium–tin oxide coated substrate showing defecting alignment. The circle indicates a highly tilted defect characteristic of the Φ_h phase. **c**, TOPM of a homeotropically aligned **D4**, obtained by cooling at $0.1^\circ\text{C min}^{-1}$ on the same substrate. Hydrophilic substrates such as plasma-treated carbon induce planar orientation of the columns.

a hydrophobic substrate from the isotropic state (Fig. 3b, c and Figure S3 of the Supplementary Information) is frozen into a glassy Φ_h state. In addition, at wide angles the XRD pattern reveals diffuse spots arranged in an X-like fashion that indicates short-range helical correlation along the column axis with an average repeat distance of $4.8 \pm 0.3 \text{ \AA}$ (E, Fig. 3a). This value arises from the packing of the aromatic regions of the dendron near the aliphatic tails. This XRD also shows diffuse spots assigned to disordered π -stacks of TNF units in the core of the column with an average separation of $3.44 \text{ \AA}$

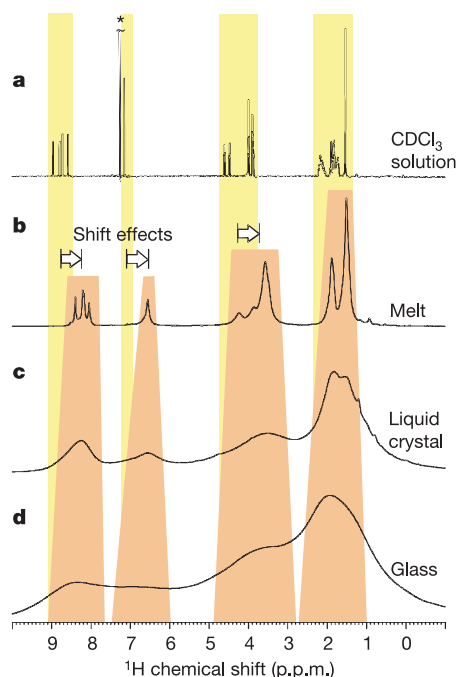


Figure 4 ^1H NMR spectra of **A1**. **a**, In CDCl_3 at 22°C ; **b**, in the isotropic melt at 120°C ; **c**, in the Φ_h liquid crystal state at 75°C ; and **d**, in the Φ_h glassy state at 25°C . Spectra **b–d** were recorded under fast magic-angle spinning. The high-field shifts caused by adjacent π -electron systems are indicated by arrows.

(C, Fig. 3a). Consequently, μ of this glassy liquid crystal state increases since the motion of the D groups from the core of the supramolecular column is decreased leading to reduced dynamic disorder (Fig. 1). Furthermore, the motion of the ionic impurities is frozen in the ordered state, whereas the photo-generated charges continue to migrate within the system. Therefore, the glassy ordered state is insensitive to ionic impurities.

Figure 4 depicts ^1H NMR spectra of **A1** in CDCl_3 solution at 22°C , in the isotropic melt at 120°C , in the Φ_h liquid crystal state at 75°C and in the Φ_h glassy state at 25°C . In this sequence, the loss of molecular mobility is reflected by the increase of the line widths. As fast magic-angle spinning is applied (except for the solution), four groups of ^1H resonances can be distinguished in all spectra: aromatic fluorenone (9–8 p.p.m.), dendron phenyl (7–6 p.p.m.), OCH_2 (4.5–3 p.p.m.) and alkyl (2.5–1 p.p.m.). In the glass, the liquid crystal phase and the melt, the lines are shifted to high field by about 0.5–0.7 p.p.m. relative to the solution-state values except for the alkyl resonances. This effect is due to aromatic π -electrons situated above or below the respective protons²⁵ and, thus, provides direct evidence for π – π packing of the fluorenones and the dendron phenyls. Analogous effects are observed in the ^1H spectra of the other materials discussed here. Moreover, in **A1** a distance of $3.5 \text{ \AA}$ was determined between the protons in adjacent fluorenone moieties by ^1H – ^1H double-quantum NMR spectroscopy²⁵, which leads to the assumption of a sandwich-type packing of pairs of nitro-fluorenone groups (Fig. 5a). Combining the NMR information with the XRD data, it can be concluded that, in the glassy Φ_h phase, the columns adopt a supramolecular structure of the form

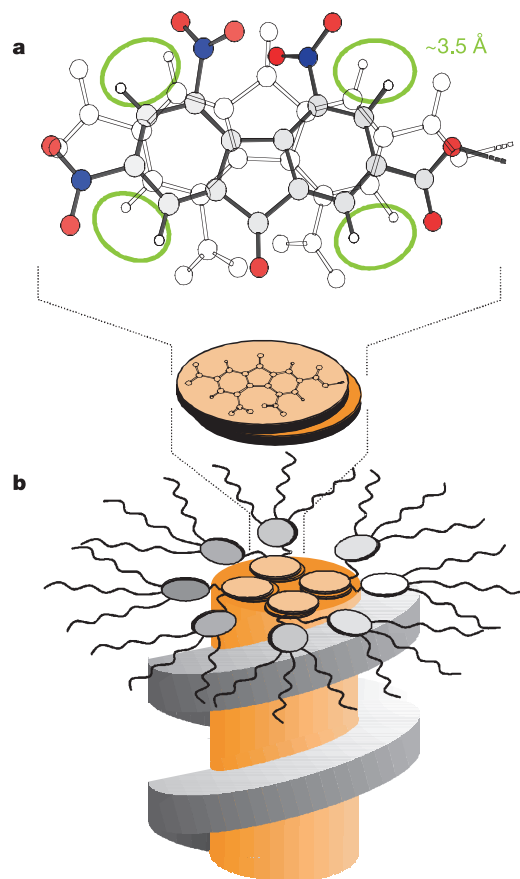


Figure 5 The molecular arrangement in the supramolecular column self-assembled from **A1**. **a**, Sandwich-type stacking of the nitro-fluorenone moieties with proton–proton distances of $3.5 \text{ \AA}$, as determined by ^1H – ^1H double-quantum NMR spectroscopy. **b**, Structure of the supramolecular columns with stacks of fluorenone sandwiches in the centre of the columns, jacketed by helical dendrons.

schematically depicted in Fig. 5b. In the centre, the fluorenone sandwiches are stacked in a column surrounded by dendrons whose phenyl groups are arranged in a helical fashion around the central column. Furthermore, NMR data indicate separation of the fluorenes (centre), dendron phenyls (inner ring) and alkyl chains (outer ring) from each other, because there are no proton–proton distances detectable between the different units on a length scale of $<4\text{ \AA}$. This clear separation is not completely achieved when the material is precipitated from solution, but only when the system is allowed to self-organize during slow cooling from the melt into the liquid crystal and glassy Φ_h phases. Before this cooling and ‘self-repair’ process, the supramolecular column contains defects introduced by intramolecular backfolded A1 dendrons.

The supramolecular arrangement shown in Fig. 5b resembles the base-pairing in DNA, which is known to support charge migration²⁸, and points to a new structural framework for incorporating dendritic molecules into functional materials²⁹. □

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Copepod hatching success in marine ecosystems with high diatom concentrations

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Diatoms dominate spring bloom phytoplankton assemblages in temperate waters and coastal upwelling regions of the global ocean. Copepods usually dominate the zooplankton in these regions and are the prey of many larval fish species. Recent laboratory studies suggest that diatoms may have a deleterious effect on the success of copepod egg hatching^{1–4}. These findings challenge the classical view of marine food-web energy flow from diatoms to fish by means of copepods^{5–7}. Egg mortality is an important factor in copepod population dynamics⁸, thus, if diatoms have a deleterious *in situ* effect, paradoxically, high

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diatom abundance could limit secondary production. Therefore, the current understanding of energy transfer from primary production to fisheries in some of the most productive and economically important marine ecosystems⁹ may be seriously flawed^{1,10}. Here we present *in situ* estimates of copepod egg hatching success from twelve globally distributed areas, where diatoms dominate the phytoplankton assemblage. We did not observe a negative relationship between copepod egg hatching success and either diatom biomass or dominance in the microplankton in any of these regions. The classical model for diatom-dominated system remains valid.

Two hypotheses have been suggested to explain the deleterious effects of diatoms on copepod reproduction in laboratory experiments. First, that diatoms are nutritionally deficient; that is, diatoms lack or are deficient in some essential component required for copepod egg development, and hence, a diatom monodiet

results in a failure of hatching¹¹. In the field, however, diatom nutritional insufficiency could be circumvented by ingestion of other prey, which would supplement the diatom diet and therefore one might not expect to see an effect. The second hypothesis proposes that diatoms are toxic^{10,12,13}; particular aldehydes have been suggested as the possible toxic agents^{1,14}. If diatoms were toxic, and in the absence of evidence of diatom avoidance by the grazers, one would expect egg hatching success to decrease when diatom concentration or relative abundance in the microplankton increases. This has been reported in one previous study¹.

In laboratory experiments conducted with copepods and cultured diatoms (16 copepod and 17 diatom species), out of 37 combinations, hatching success was significantly reduced in 29 (ref. 2). We have adapted this approach to natural systems and we have conducted a series of field experiments under natural conditions. In these experiments we measured both copepod egg hatching success and diatom concentration in areas globally representative of ecosystems where diatoms dominate the microplankton: the Oregon coastal upwelling, Labrador Sea, Scotian Shelf, lower St Lawrence estuary, Georges Bank, Long Island Sound, Iceland Basin, three northern Norwegian fjords, the English Channel, the northern Adriatic, the Benguela coastal upwelling system off Namibia, and in the Southern Ocean off South Georgia. Experiments were performed with 13 species of calanoid copepods, while diatom concentrations ranged from 0.05 to 2,500 mg C m⁻³ (equivalent to 0.1–57 mg chlorophyll *a* m⁻³) and comprised 0.2–99% of the microplankton biomass (>2 µm cells).

From a total of 294 observations, 76% of these showed hatching success exceeding 80% (Fig. 1). Although low hatching success was observed occasionally (in only 8% of the observations was the hatching success below 50%), for none of the areas or species did we find a significant relationship between hatching success and diatom biomass (Fig. 1a), or relative contribution to the total microplankton biomass (Fig. 1b). Nor did we find a relationship when all the data were considered together (Fig. 1a, b) (see Supplementary Information).

There are a number of possible explanations for the differences between the laboratory and the field results. The first is that diatoms may not be toxic, but merely nutritionally deficient¹¹. A second explanation is the timescale and food concentration in the experiments. In the laboratory, the deleterious effects generally appear after one to two weeks feeding on a diatom monodiet. This situation is unlikely in the field because peak concentrations during diatom blooms are not maintained for periods of weeks and because female longevity in the field is generally in the order of days rather than weeks¹⁵. Most of the laboratory experiments showing deleterious effects of diatoms have been undertaken at very high diatom concentrations (over 1,000 µg C l⁻¹). In contrast, our survey of diatom concentrations in some of the most productive regions globally, shows that such high concentrations are extremely rare; only on one occasion did we observe a concentration exceeding 1,000 µg C l⁻¹ (lower St Lawrence, 2,449 µg C l⁻¹). However, even in this case, the hatching success remained high (95%). Our results also raise a general issue about the applicability of simplified laboratory food suspensions to the complexity of natural assemblages. Copepods often select microzooplankton (for example, ciliates) over phytoplankton, so that non-diatom food can represent a higher percentage of the diet than occurs in the natural microplankton assemblage. Avoidance of diatoms in the field could also explain the differences between laboratory and field results; however, both laboratory experiments and field measurements show that copepods do eat diatoms and often select them actively over other phytoplankters¹⁶. Furthermore, both in the laboratory and in the field, egg production generally increases with increasing diatom concentration^{2,17}. The other possible explanations for the differences between the laboratory and the field results relate to the diatom assemblage and culture conditions; that is, either that the

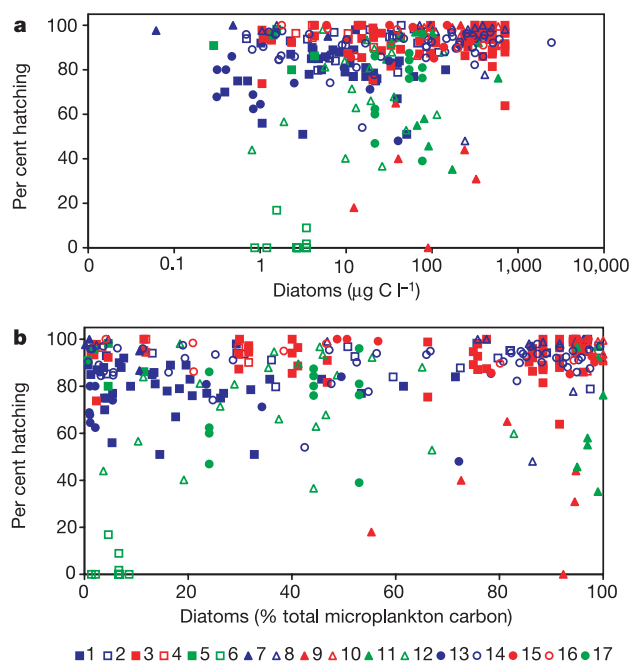


Figure 1 Diatoms and copepod egg hatching success. **a**, Copepod egg hatching success as a function of diatom carbon concentration. **b**, Copepod egg hatching success as a function of the proportion of diatom carbon in the total microplanktonic community. Measurements were: (1) for *Calanus helgolandicus* in the English Channel (50° 15' N, 4° 13' W) weekly observations from January to September 1994 and from March to July 2001, *n* = 36; (2) for *Calanus finmarchicus* on the Scotian Shelf (42° 45' N to 47° 10' N and 58° 10' W to 65° 29' W) during April 1997, *n* = 9, and in the Labrador Sea (54° 30' N to 60° 22' N and 48° 30' W to 54° 45' W) during May 1997, *n* = 12. (3) for *Calanoides carinatus*, *n* = 63, (4) *Centropages brachiatus*, *n* = 5, (5) *Metridia lucens*, *n* = 5, (6) *Nannocalanus* sp., *n* = 10, (7) *Pleuromamma* sp., *n* = 5 and (8) *Rhincalanus nasutus*, *n* = 13, all in the Benguela coastal upwelling (between 28° 40' S and 25° 30' E and between 11° 10' S and 14° 50' E) during October 2000; (9) for *Temora longicornis* in Long Island Sound (41° 17' N, 72° 2.7' W) weekly from February to March 2001, *n* = 7; (10) for *Rhincalanus gigas*, *n* = 10 and (11) for *Calanoides acutus*, *n* = 8, off South Georgia (54° S, 38° W) during December 2000 and January 2001; (12) for *C. finmarchicus* in the Iceland Basin (around 60° N, 15° W) during April 2001, *n* = 22; (13) for *C. finmarchicus* on Georges Bank (40° 48' N, 68° W) during April–May 1997, *n* = 12; (14) for *C. finmarchicus* in the lower St Lawrence estuary (48° 40' N, 68° 35' W) observations were made weekly from April to September 1997, May to October 1998 and 1999, and from May to August 2000, *n* = 50; (15) for *Acartia clausi* in the northern Adriatic (45° 42. 06' N, 13° 42.6' E) in May 1999, January–March, May, August and December 2000, and February and May 2001, *n* = 8; (16) for *Calanus marshallae* Oregon coastal upwelling (44° 40' N, 124° 10.6' W) in May, June and July 2001, *n* = 7; (17) for *C. finmarchicus* in the fjords around Trømsø (69° 22' N, 19° 07' E) in April 2001, *n* = 12.

toxicity is due to the diatom culture conditions in the laboratory or that cases of toxicity are exceptions, owing to the species or strains maintained in laboratory cultures being unrepresentative of natural field populations.

However, any explanation for the discrepancy between the laboratory and field results does not affect our conclusion. The range of areas and copepod and diatom species considered in this study provide strong evidence that, under natural environmental conditions, there is no negative effect of diatoms on copepod hatching success. We conclude that there is no need to revise existing conceptual models of energy transfer from phytoplankton, through copepods, to fish in diatom-dominated systems. □

Methods

Hatching success

Eggs for the hatching success measurements were obtained from females incubated in filtered or natural sea water (depending on the species, some copepods stop spawning in filtered sea water) during the first 12–24 h after capture¹⁸. The intention was to minimise the effect of the incubation conditions to obtain hatching rates representative of the field values. From the egg production experiments 30–100 eggs were selected randomly and gently transferred to 60-ml tubes filled with filtered sea water. The samples were incubated, at sea surface temperature, for periods ranging from 48 to 96 h (depending on the temperature). After the incubation period, the samples were examined microscopically to determine the number of nauplii and unhatched eggs.

Microplankton identification and biomass

Water samples for identification of microplankton (>2 µm, nanoplankton plus microplankton) species and carbon estimation were collected generally at the chlorophyll maximum depth and preserved with 1% final concentration of Lugol's iodine solution¹⁹. Subsamples (100 ml) were settled (Utermöhl technique) and counted with an inverted microscope. Phytoplankton carbon biomass was estimated from cell volume²⁰ and using a factor of 0.21 pg C µm⁻³ (ref. 21) for ciliates. Heterotrophic dinoflagellates were separated from autotrophic forms according to taxonomic considerations²².

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Epiparasitic plants specialized on arbuscular mycorrhizal fungi

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Over 400 non-photosynthetic species from 10 families of vascular plants obtain their carbon from fungi and are thus defined as myco-heterotrophs¹. Many of these plants are epiparasitic on green plants from which they obtain carbon by 'cheating' shared mycorrhizal fungi^{2–7}. Epiparasitic plants examined to date depend on ectomycorrhizal fungi for carbon transfer and exhibit

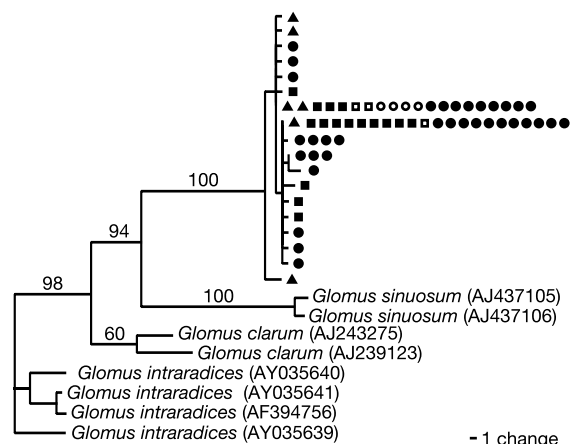


Figure 1 The AMF of *Arachnitis* are markedly similar, showing minimal variation in the generally highly polymorphic internal transcribed spacers of nuclear DNA. The phylogenetic tree shows the placement of fungal sequences among their closest available alignable relatives. Identical sequences are represented by symbols in a row: filled triangles, circles, and squares correspond to fungal sequences obtained from root samples of *Arachnitis* from each of three locations (eight plants); open symbols correspond to fungal sequences obtained from adjacent root samples of green plants that are identical to sequences obtained from *Arachnitis* root samples. Neighbour-joining tree with 10,000 bootstrap replicates for 71 sequences and 442 characters. GenBank accession numbers are shown in parentheses.

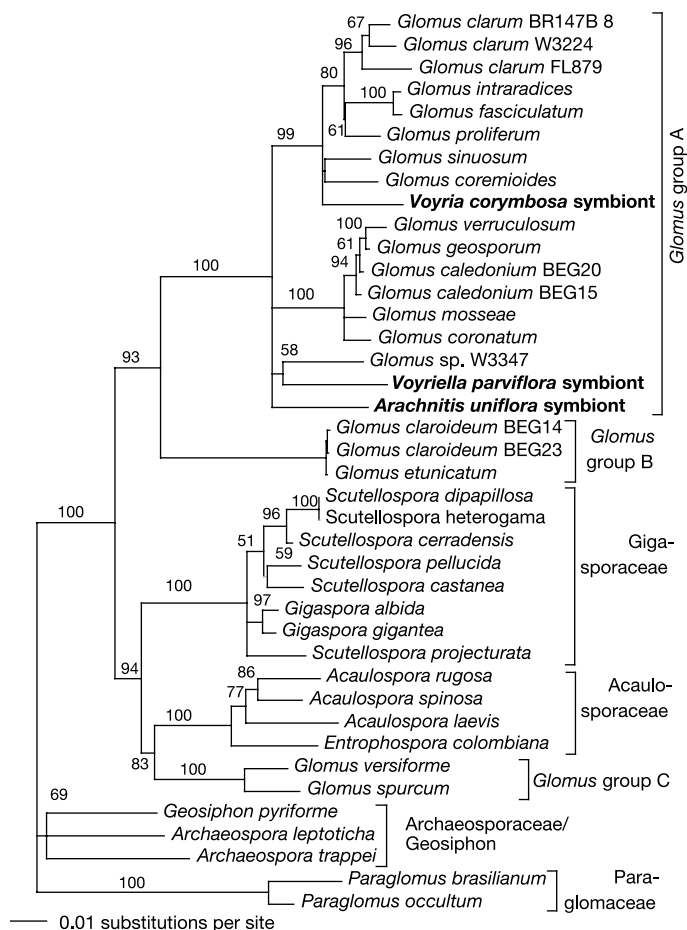
exceptional specificity for these fungi^{3–7}, but for most myco-heterotrophs neither the identity of the fungi nor the sources of their carbon are known. Because many myco-heterotrophs grow in forests dominated by plants associated with arbuscular mycorrhizal fungi (AMF; phylum Glomeromycota), we proposed that epiparasitism would occur also between plants linked by AMF. On a global scale AMF form the most widespread mycorrhizae, thus the ability of plants to cheat this symbiosis would be highly significant. We analysed mycorrhizae from three populations of *Arachnitis uniflora* (Corsiaceae, Monocotyledonae), five *Voyria* species and one *Voyriella* species (Gentianaceae, Dicotyledonae), and neighbouring green plants. Here we show that non-photosynthetic plants associate with AMF and can display the characteristic specificity of epiparasites. This suggests that AMF mediate significant inter-plant carbon transfer in nature.

Arbuscular mycorrhizal fungi (AMF) are nutrient-gathering obligate symbionts of most plant species. In contrast to the situation in the ectomycorrhizal symbiosis, the arbuscular mycorrhizal symbiosis lacks examples of absolute specificity and net fungus-to-plant carbon transfer^{3,8–10}. Despite the widespread potential for inter-plant linkage offered by the generalist behaviour of AMF^{10–12}, experimental evidence for inter-plant carbon transfer is equivocal because transferred carbon may remain in fungal structures within

roots and carbon flux between autotrophs may be bi-directional^{13,14}. Consequently, it has been stated that in nature “there is no fungus-to-plant carbon transfer”¹⁴. However, by definition, net carbon transfer must occur from fungus to myco-heterotroph. Although in the ectomycorrhizal symbiosis myco-heterotrophs are extreme examples of both specialization and epiparasitism, evidence for these phenomena is lacking in putative arbuscular mycorrhizal myco-heterotrophs^{1,15,16}.

We show that the non-photosynthetic plant *Arachnitis uniflora* from three subantarctic forest sites in Argentina forms arbuscular mycorrhizae and that it is specialized to a narrow lineage within a clade of *Glomus* (*Glomus* group A¹⁷). We were able to use universal fungal-specific primers to amplify and sequence the highly polymorphic nuclear ribosomal internal transcribed spacer (nrITS) region directly from *Arachnitis* roots. This in itself is unusual, because polymerase chain reaction (PCR) detection of AMF in green plants usually requires specific primers, nested amplifications, and cloning due to low template concentrations, inhibitors, and high complexity of root fungal communities. The sequences from all eight specimens were nearly identical. To verify the absence of other mycorrhizal fungi, we cloned and sequenced from the direct amplicon pools of all specimens (Fig. 1). The nrITS sequences obtained show a polymorphism that is within the range of intra-

a Complete 18S rDNA tree



b Partial 18S rDNA trees

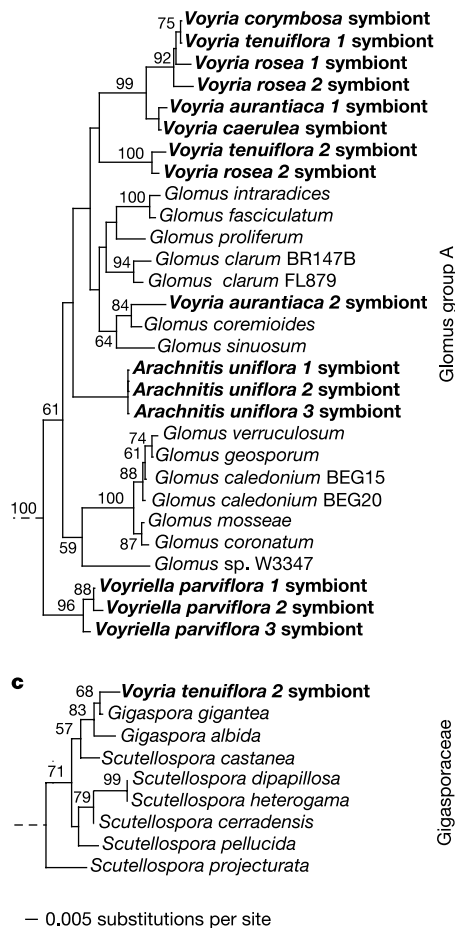


Figure 2 Most of the arbuscular mycorrhizal symbionts of the three plant genera sampled fall into three distinct clades within *Glomus* group A. **a**, Phylogenetic tree using complete 18S nuclear DNA sequences from all known families and lineages of AMF and a subset of myco-heterotroph symbionts. Neighbour-joining tree using 1,611 characters. Nodes with bootstrap values below 50 were collapsed to polytomies. **b**, *Arachnitis* AMF are found only in one narrow clade of *Glomus* group A. Three of eight nearly identical AMF 18S nuclear DNA sequences obtained from three plants are shown here, one from each site sampled.

Voyriella AMF are restricted to another *Glomus* group A lineage, whereas *Voyria* spp. have the most diverse set of AMF within *Glomus* group A. Neighbour-joining tree with 1,000 bootstrap replicates using 450 characters from the 3'-terminal region of the 18S. *Glomus* group B was used as an outgroup. **c**, The only detected *Voyria* symbiont not in *Glomus* group A is in the Gigasporaceae. This tree was obtained as in **b**, with *Acaulospora laevis* used as an outgroup.

species or intra-spore variation in AMF¹⁸. The *Glomus* lineage detected in *Arachnitis* also formed mycorrhizae in adjacent photosynthetic plants from three different families (Fig. 1); these or other co-occurring plants must be the ultimate sources of carbon for *Arachnitis*, as AMF are not saprotrophs. The plant roots were identified morphologically or by nrITS sequence similarity to GenBank accessions or leaves obtained at the sites: *Osmorhiza chilensis* (Apiaceae) (AF480090), *Austrocedrus chilensis* (Cupressaceae), and *Nothofagus dombeyi* (Nothofagaceae) (AF480091, AF480092). We detected three other AMF lineages in the vicinity of *Arachnitis* roots but not associated with them, indicating that plant specificity is not due to a lack of other co-occurring AMF. Furthermore, despite the presence of ectomycorrhizal autotrophs throughout its geographical range, *Arachnitis* has not invaded the ectomycorrhizal mutualism as have other epiparasitic monocots (that is, orchids^{4,5}). Instead, the arbuscular mycorrhizae-forming ancestors of *Arachnitis* reversed the direction of carbon flow entirely in their favour, and specialized on a narrow subset of AMF.

Voyriella and *Voyria* samples from tropical rainforests in French Guyana were also associated with a restricted set of closely related AMF. The intraradical morphology of the fungal symbionts of *Voyria* has been interpreted as a special form of arbuscular mycorrhiza^{1,15}, but proof was lacking because arbuscules are not formed. We show that the AMF of *Voyriella* and most *Voyria* examined are from the same clade of *Glomus* as the AMF of *Arachnitis*. All AMF of *Voyriella* were from one narrow lineage within *Glomus* group A. Most AMF of *Voyria* (*V. aurantiaca*, *V. rosea*, *V. caerulea*, *V. corymbosa*, *V. tenuiflora*) formed a monophyletic lineage within *Glomus* group A (Fig. 2). The only exceptions were one *V. aurantiaca* root containing a symbiont related to *Glomus sinuosum* and a *V. tenuiflora* root with both *Gigaspora* and the *Glomus* we found in all the other *Voyria* species. The principal lineage associated with *Voyria* was also detected in adjacent photosynthetic plant roots, including roots near *Voyriella*. Furthermore, there was no symbiont overlap between *Voyria* and *Voyriella* at a site where they co-occurred. Therefore, as in ectomycorrhizal epiparasites^{4,6,7}, the specificity observed is not based solely on local availability of susceptible fungi.

These are currently the only known examples of specialization by a plant for a phylogenetically restricted set of AMF. Parasites are generally more specialized than mutualists¹⁹, and accordingly both ectomycorrhizal and now arbuscular mycorrhizal epiparasites turn out to be more specialized than their photosynthetic counterparts. Epiparasitism is at the extreme end in a continuum of plant–fungal mycorrhizal interactions that ranges from parasitism of either partner to mutualistic interactions^{20,21}. This suggests that we should look for evidence of inter-plant carbon transfer among pairs of green plants that have been found to differ in species-specific outcomes of AMF colonization^{22–25}, as specialized myco-heterotrophy suggests that net flux of carbon from fungus to plant occurs within arbuscular mycorrhizal networks. □

Methods

Arachnitis fungi

We obtained eight plants of *Arachnitis uniflora* from three populations in Nahuel Huapi National Park (Rio Negro province, Argentina): two from Lake Verde, one from Mount Otto, and five from the Llao-Llao area (41° 7.27' S, 71° 23.52' W). Lake Verde is approximately 75 km away from Llao-Llao, and Mount Otto is halfway between these two locations. The plants encompassed the morphological variation (that is, size and colour) observed at these sites and all produced an identical plant nrITS sequence (AF480089). For microscopy, *Arachnitis* roots were embedded (Kulzer Histo-Technique 7100), sectioned (4 µm) and stained with Cresyl blue, Cotton blue, Trypan blue, or iodine solution. The fungal structures are characteristic of vesicular arbuscular mycorrhizae and include intracellular bundles of 5–6 vesicles borne from a single hypha, coils, and relatively sparse arbuscules. When the flowering shoot develops, colonized cortical layers become completely lysed and only loosely arranged mycelium remains.

We also extracted genomic DNA from individual sections (~0.5 mm × 2 mm) of roots from each plant following methods described elsewhere²⁶, with a purification step using GeneClean II (Q-Biogene). We amplified the fungal nrITS using the universal fungal primers ITS1F and ITS4 (refs 26, 27) and we sequenced 6–12 fungal nrITS clones from each plant (AF480093–AF480148). About 15% of these sequences were disregarded as they

were plant ITS sequences or sequences closely related to fungal lineages known to not form mycorrhizae (*Neurospora*, *Rhodoturula*, *Mortierella*). The complete 18S was cloned and sequenced from NS1 and GLOM5.8R^{27,28} amplicons obtained from one plant from each location (AF480150–AF480158).

Fungal symbionts of plants near *Arachnitis*

To test whether the *Arachnitis* mycorrhizal fungus is also present in roots of photosynthetic plants, we designed a primer specific to the symbiont (glomits1f: GCGTCCGTCATTATTT AAAACC). From 36 root fragments (5 mm long) collected near or attached to *Arachnitis* roots we extracted genomic DNA and amplified the nrITS with glomits1f and ITS4. Three root fragments amplified in the first round of PCR, and an additional four amplified after a second round using 10^{–2} dilutions of the initial reactions. These amplicons were sequenced to confirm their identity as the *Arachnitis* symbiont (AF503648–AF503654). The genomic extracts of the roots were also used to amplify the plant nrITS using the primers ITS1 and ITS4. To assess some of the diversity of other AMF near *Arachnitis* roots, we applied a clade-specific nested PCR approach²⁹ to the same roots of photosynthetic plants used above and we found two different nuclear DNA sequences from *Glomus* groups A and B (AF480160, AF480161). In addition, a sporocarp with another nuclear ribosomal DNA sequence from *Glomus* group A (AF480159) was found less than 10 cm from one *Arachnitis*. These three sequences did not match the *Arachnitis* symbiont sequences and were too divergent for unambiguous alignment to the data set analysed in Fig. 1.

Voyria and *Voyriella* fungi

We sampled three plants of *Voyriella parviflora*, two of *Voyria rosea*, two of *V. tenuiflora* and one of *V. corymbosa* from a site in central French Guyana (3° 40.4' N, 53° 12.5' E). One *V. caerulea* and two *V. aurantiaca* plants were sampled from another site about 10 km away. Genomic DNA was extracted using a Qiagen DNeasy mini kit. A nested PCR procedure described elsewhere²⁸ was used to amplify the nrITS and the 3'-terminal region of the 18S ribosomal DNA. The fungal symbionts were members of *Glomus* group A based on NS5 and GLOM5.8R PCR products, which were cloned, screened with *Hinf*I, and sequenced (AJ430854–AJ430858, AJ437204–AJ437208, AJ437210). In one case, a Gigasporaceae symbiont was identified using the primers NS5 and GIGA28R (AJ437216, GIGA28R: TTCAGCGGGTACTCTACA). In a subset of specimens the less specific primer AM1 (ref. 12) used in combination with NS3 (ref. 27) confirmed our results (AJ437211–AJ437215) and the absence of other AMF. Combinations of universal primers, AM1, and primers specific to *Glomus* group A (GLOM1070R²⁹, GLOM5.8R²⁸, GLOM1311R: GAAGCTGGCGACCTAACA) were used to amplify and sequence the complete 18S of the two principal fungal symbionts (AJ430852, AJ430853). Most *Glomus* group A sequences obtained fell into two types: type 1 was detected in all plants of *Voyriella* and type 2 was found in all *Voyria* plants. A third type, related to *Glomus sinuosum*, was found in one plant of *V. aurantiaca*. The roots of other plants intermingled with those of *Voyria* and *Voyriella* were analysed by the same methods and type 2 sequences were detected (AJ437209, AJ438773). In addition, we designed primers specific to type 1 (voyM1660: TGTCAAGGGTCTTTGGTTGG) and type 2 (voyQ1660: ATTAGGACTGGMAACA GAC). Type 1 products were successfully amplified only from *Voyriella* and type 2 only from *Voyria*. These amplicons were screened by restriction analysis and their identity confirmed by sequencing (AJ437102, AJ437103).

DNA sequence analysis

Nucleotide sequencing was performed on ABI 310 and ABI 3100 Genetic Analyzers using BigDye chemistry. Sequences were aligned manually into datasets of: (1) internal transcribed spacer sequences from symbionts and *Glomus* group A sequences from the database; (2) partial 18S nuclear DNA sequences³²; and (3) sequences of the complete 18S nuclear DNA of glomalean fungi and outgroups from the database. Data sets were analysed with PAUP*4.0beta8 (ref. 30).

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The authors declare that they have no competing financial interests.

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RGM is a repulsive guidance molecule for retinal axons

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Axons rely on guidance cues to reach remote targets during nervous system development¹. A well-studied model system for axon guidance is the retinotectal projection. The retina can be divided into halves; the nasal half, next to the nose, and the temporal half. A subset of retinal axons, those from the temporal half, is guided by repulsive cues expressed in a graded fashion in

the optic tectum^{2,3}, part of the midbrain. Here we report the cloning and functional characterization of a membrane-associated glycoprotein, which we call RGM (repulsive guidance molecule). This molecule shares no sequence homology with known guidance cues, and its messenger RNA is distributed in a gradient with increasing concentration from the anterior to posterior pole of the embryonic tectum. Recombinant RGM at low nanomolar concentration induces collapse of temporal but not of nasal growth cones and guides temporal retinal axons *in vitro*, demonstrating its repulsive and axon-specific guiding activity.

The retinotectal map is characterized by a precise mapping of retinal ganglion cells to the optic tectum so that their terminations form a reversed image of the retina². The temporal half of the retina projects to the anterior part of the optic tectum, and the nasal retina to the posterior tectum². Glycosylphosphatidylinositol (GPI)-linked molecules have been proposed to be important in guiding temporal retinal axons to their correct topographic position in the optic tectum by repelling this axon class from the posterior part of the tectum³. Efforts to identify these guidance cues led to ephrins^{4,5} (that is, ligands of Eph receptor tyrosine kinases), and to a GPI-anchored glycoprotein that had a relative molecular mass of 33,000 (M_r 33K) and an isoelectric point of approximately 8 (ref. 6).

On the basis of these characteristics, we purified a protein from tectal membranes of embryonic chicken, and analysed it by nano-electrospray mass spectrometry. Subsequent screening of a complementary DNA library from embryonic chicken with a probe derived from the resulting amino-acid sequences retrieved a cDNA of 1,486 nucleotides (Fig. 1a). The protein encoded consists of 432 residues; it covered 9 out of ten sequence stretches obtained from mass spectrometry (Fig. 1a). The methionine indicated in Fig. 1a probably defines the translation start of RGM as it is preceded by a Kozak sequence⁷. Surprisingly, the predicted relative molecular mass for a protein with 432 residues is much higher than 33K. Indeed, an amino-terminal sequence determination using Edman degradation resulted in the amino-acid sequence PHLRT, strongly suggesting that native RGM starts with residue 150 (Fig. 1a).

Sequence analysis showed that RGM has no significant homology to any other known guidance molecule. As predicted by the hydrophobicity profile using the Kyte and Doolittle algorithm⁸, RGM contains two hydrophobic domains at the N and carboxy terminus (Fig. 1b, c). The N-terminal domain seems to represent a conventional signal peptide, whereas the C-terminal domain is a GPI-anchor domain, in agreement with the characterization of RGM as a GPI-anchored plasma membrane protein⁶. Moreover, there is a tri-amino-acid motif, the RGD site⁹, which could be involved in cell attachment, and a partial von Willebrand factor type D domain¹⁰. Most of its key amino acids are present in RGM. Nevertheless, it is only a partial domain, comprising about 40% of a full-length domain¹⁰.

We investigated the distribution of RGM message and protein expression by *in situ* hybridization and antibody staining. *In situ* hybridization with an RGM-specific anti-sense probe on cryostat sagittal sections from chicken tecta (E9) showed strong staining in the periventricular layer surrounding the tectal ventricle (Fig. 2a). The staining intensity observed with an RGM anti-sense probe is much stronger in posterior than anterior tectum. This staining gradually increases from the anterior to posterior pole, suggesting that RGM mRNA forms a spatial gradient along the anterior-posterior axis of the embryonic chicken tectum. DAPI staining of the nuclei revealed that the cell density in the periventricular layer is almost constant along the anterior-posterior axis (Fig. 2b). This finding implies that the gradient is formed by a different degree of expression rather than by a different density of cells expressing RGM mRNA.

The polyclonal antibody raised against chicken RGM specifically recognized a single protein of M_r 33K on western blots (Fig. 2c). To

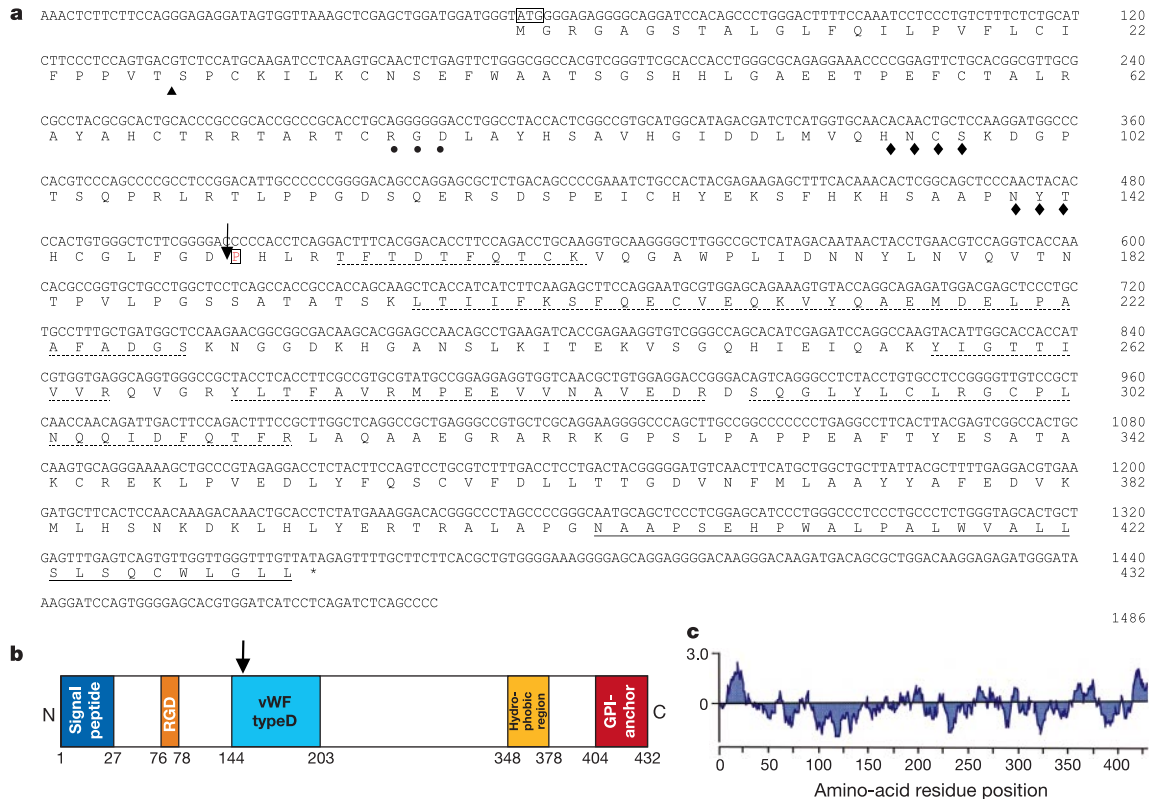


Figure 1 RGM sequence, domain model, and hydrophobicity profile. **a**, Nucleic-acid and amino-acid sequences of chicken RGM. Box, presumed initiation codon; triangle, potential signal peptide cleavage site; dots, RGD site; diamonds, predicted N-glycosylation sites; arrow, cleavage site; residues marked by a dotted line, peptides

analysed by mass spectrometry; underlining, GPI-anchor sequence; asterisk, stop codon. **b**, Main structural features of RGM protein. vWF, von Willebrand factor. The arrow marks the proteolytic cleavage site. **c**, Hydrophobicity plot (Kyte-Doolittle units averaged over 9 residues).

study RGM characteristics in embryonic tecta, we treated anterior and posterior tectal membranes with phosphatidylinositol-dependent phospholipase C (PI-PLC). The resulting supernatants were analysed by SDS gel electrophoresis and immunoblotting. RGM is specifically removed from tectal membranes by PI-PLC (Fig. 2c), indicating that it is linked to the membrane by way of a GPI anchor. Non-GPI linked membrane proteins, such as NgCAM, are not removed by treatment with PI-PLC¹¹. Supernatants derived from posterior tectal membranes contain a significantly higher amount of RGM than those from anterior membranes, strongly suggesting that both RGM mRNA (Fig. 2a) and protein (Fig. 2c) are enriched in the

posterior optic tectum.

For analysis of RGM function *in vitro*, we used the collapse assay¹². In this assay, the motile tips of the axons, the growth cones, are confronted with target-derived membranes. Temporal retinal growth cones collapsed when confronted with membranes containing RGM, whereas no collapse was observed with mock-transfected cells used as controls (Fig. 3a). Dose response curves revealed that temporal growth cones are much more sensitive to RGM than nasal growth cones (Fig. 3a, b). In contrast to temporal growth cones, nasal cones do not collapse when confronted with RGM at low nanomolar concentration (Fig. 3b). As a control, we analysed the

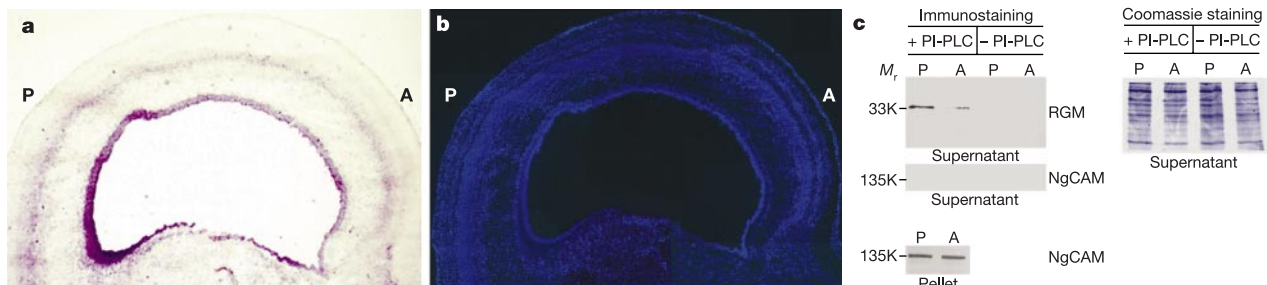


Figure 2 Graded expression of RGM in the embryonic optic tectum. **a**, *In situ* hybridization of embryonic chicken tectum with an RGM-specific anti-sense probe shows intense staining of the periventricular layer and a weaker staining of a more superficial layer. The staining in the periventricular layer gradually increases from anterior (A) to posterior (P). **b**, Nuclei staining of the same tectum. **c**, An RGM-specific antibody recognizes a 33K

protein which is removed from tectal membranes by phosphatidylinositol-dependent phospholipase C (PI-PLC) and more abundant in posterior than anterior tectal membranes. The non-GPI linked membrane protein NgCAM (pellet) is not removed by PI-PLC (Supernatant).

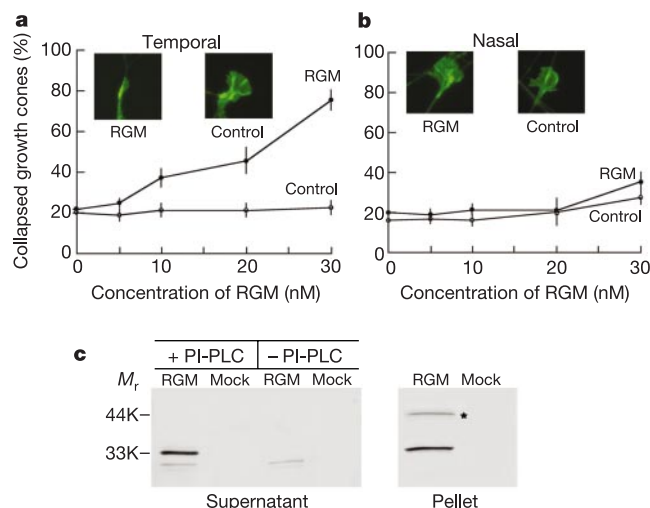


Figure 3 Collapse of temporal retinal growth cones induced by RGM. **a**, Temporal retinal growth cones are confronted with membranes prepared from either RGM- or mock-transfected HEK 293 cells. Membranes containing RGM at low nanomolar concentration induce collapse of temporal growth cones. **b**, Nasal growth cones are resistant to RGM at low concentration, but collapse when confronted with RGM at high concentration (exceeding 30 nM). Data are from four independent experiments. **c**, Membranes from transfected cells were treated with PI-PLC, resulting in the removal of RGM. Asterisk, RGM precursor.

membranes used in this study by incubating them with PI-PLC (Fig. 3c). RGM is specifically removed by PI-PLC, demonstrating the presence of a GPI-anchor in recombinant RGM. Ephrins were not detectable in the membranes of HEK 293 cells transfected with RGM cDNA (data not shown). We conclude that the observed collapse-inducing activity is solely due to RGM.

We next examined whether recombinant RGM is able to guide retinal axons *in vitro*. To address this question, we performed the well-characterized stripe assay². In this assay, growth cones are offered two kinds of stripes containing different target-derived membranes, that is, membranes from RGM- and mock-transfected

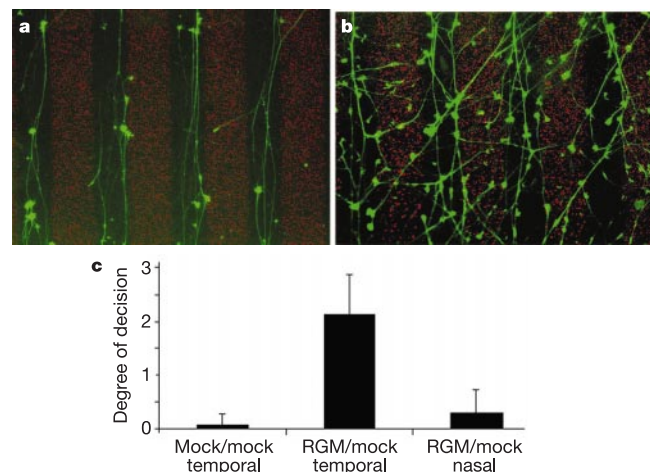


Figure 4 Guidance of temporal retinal axons by RGM. **a**, Temporal retinal axons avoid stripes (labelled with red fluorescent beads) derived from membranes of RGM-transfected HEK 293 cells. The unlabelled stripes contain membranes from mock-transfected HEK 293 cells. **b**, Nasal retinal axons do not show any avoidance reaction on these membrane carpets. **c**, Analysis of the degree of preference.

HEK 293 cells. On these 'carpets', temporal but not nasal axons made a clear decision against RGM (Fig. 4a, b). No decision was observed on carpets prepared from membranes of only mock-transfected cells (data not shown). Using the method of ref 2 for evaluation, we obtained the following degrees of preference (Fig. 4c): 2.2 for temporal axons ($n = 38$), and 0.3 for nasal axons ($n = 38$). The repulsiveness is confined to a subset of retinal axons, those from the temporal half of the retina, clearly demonstrating that RGM acts as an axon-specific repulsive guidance molecule.

Our study identifies RGM, a GPI-linked axon guidance molecule of the retinotectal system. RGM is repulsive for a subset of axons, those from the temporal half of the retina. Temporal retinal axons invade the anterior optic tectum in a superficial layer, and encounter RGM expressed in a gradient with increasing concentration along the anterior-posterior axis. Temporal axons are able to receive posterior-dependent information by sensing gradients or concentrations of guidance cues¹³. Thus, RGM is likely to provide positional information for temporal axons invading the optic tectum in the stratum opticum. The present identification and characterization of RGM is important for our understanding of retinotectal map formation, and will help to elucidate the molecular mechanisms of axon guidance. □

Methods

Protein purification and nanoelectrospray mass spectrometry

Conventional procedures to purify RGM to homogeneity failed. Therefore, we decided to prepare tectal membranes from 2,000 chicken embryos (E9/10) and to microsequence all proteins with the following characteristics: sensitivity to the enzyme PI-PLC indicating a GPI-anchor, M_r between 30K and 35K, and isoelectric point between 7 and 9. For this purpose, proteins sensitive to treatment with PI-PLC were separated by two-dimensional gel electrophoresis and stained with silver. Only one protein spot fulfilled all three requirements. It had the same biochemical properties as reported previously⁶. The RGM spot was visible in a former study, but was overlooked because of its low abundance⁶. In the present study, the 33K spot was cut out, digested with trypsin and the resulting peptides analysed by nanoelectrospray tandem mass spectrometry¹⁴. Detailed examination of the mass spectra resulted in ten partial peptide sequences.

Screening of a cDNA library, *in situ* hybridization and generation of antibodies

Three out of ten peptide sequences obtained by mass spectrometry were used for synthesis of degenerate oligonucleotide primers. Combinations of forward and reverse primers resulted in the polymerase chain reaction (PCR) amplification of a 459-base-pair fragment. This fragment served as probe to screen an E14 chicken brain cDNA library. *In situ* hybridization on cryostat sections of the chicken tectum and DAPI staining were performed as described⁵. A chicken RGM peptide spanning residues 279 to 289 was used to generate rabbit antibodies.

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Competing interests statement

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Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency

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Apoptosis is a form of programmed cell death that is controlled by aspartate-specific cysteine proteases called caspases. In the immune system, apoptosis counters the proliferation of lymphocytes to achieve a homeostatic balance, which allows potent responses to pathogens but avoids autoimmunity^{1,2}. The CD95 (Fas, Apo-1) receptor triggers lymphocyte apoptosis by recruiting Fas-associated death domain (FADD), caspase-8 and caspase-10 proteins into a death-inducing signalling complex^{3,4}. Heterozygous mutations in CD95, CD95 ligand or caspase-10 underlie most cases of autoimmune lymphoproliferative syndrome (ALPS), a human disorder that is characterized by defective lymphocyte apoptosis, lymphadenopathy, splenomegaly and autoimmunity^{5–14}. Mutations in caspase-8 have not been described in ALPS, and homozygous caspase-8 deficiency causes embryonic lethality in mice. Here we describe a human kindred with an inherited genetic deficiency of caspase-8. Homozygous individuals manifest defective lymphocyte apoptosis and homeostasis but, unlike individuals affected with ALPS, also have defects in their activation of T lymphocytes, B lymphocytes and natural killer cells, which leads to immunodeficiency. Thus, caspase-8 deficiency in humans is compatible with normal development and shows that caspase-8 has a postnatal role in immune activation of naive lymphocytes.

Some individuals studied at the NIH under approved protocols manifest ALPS-related clinical features but do not have mutations in CD95, CD95 ligand or caspase-10 (ref. 15). Among them, we identified two siblings in family 66, a 12-yr-old female (patient 1)

and an 11-yr-old male (patient 2), who showed lymphadenopathy, splenomegaly and defective CD95-induced apoptosis of peripheral blood lymphocytes (PBLs; Table 1 and Fig. 1a). Unlike typical individuals affected with ALPS, they had immunodeficiency characterized by recurrent sinopulmonary and herpes simplex virus (HSV) infections and poor responses to immunization (Table 1). The affected siblings were developmentally normal. The unaffected mother, father and sister were clinically well, although their PBLs showed partial defects in apoptosis mediated by CD95 (Fig. 1a and data not shown). Cells from patient 1 responded normally to the mitochondrial apoptosis inducers staurosporine, ceramide and etoposide (Fig. 1b).

Biochemical analysis of the death-inducing signalling complex (DISC) showed normal CD95-induced FADD recruitment in all members of family 66 examined (Fig. 1c and data not shown). By contrast, the cellular abundance of caspase-8 and its recruitment to the DISC were decreased markedly in patient 1 and patient 2, but were normal in the mother (Fig. 1c, d, and data not shown). Neither caspase-8 nor the downstream effector caspase-3 underwent cleavage after Fas ligation, indicating that the DISC from patient 1 cells was not functional (Fig. 1e). We sequenced the complementary DNAs of CD95, CD95 ligand, caspase-10 and FADD from family members but found no mutations; however, we identified a homozygous C to T mutation in caspase-8 in both patient 1 and patient 2 that changed an arginine to a tryptophan at residue 248 in the p18 protease subunit (Fig. 2a).

The asymptomatic mother, father and sister were heterozygous carriers of this mutation. We screened 13 extended family members and detected seven asymptomatic heterozygous carriers, but no additional homozygous or immunodeficient individuals. The pedigree revealed consanguinity (Fig. 2b). We did not find the mutation in DNA samples from 40 normal donors and 40 individuals affected with systemic lupus erythematosus, indicating that the Arg248Trp mutation is not a polymorphism. No additional caspase-8 mutations were found in 10 individuals with common variable immunodeficiency or 30 individuals with ALPS-like conditions (data not shown).

We examined the activity of the mutant enzyme. Using purified chimaeric proteins of wild-type or mutant caspase-8 protease fused to glutathione S-transferase (GST), we found that the mutant protein was unable to cleave the reporter substrate DEVD-AMC (Fig. 2c). In a cellular reconstitution assay using a caspase-8-deficient Jurkat cell line (19.2) that fails to undergo apoptosis after CD95 crosslinking, expression constructs encoding wild-type caspase-8 restored CD95-induced apoptosis, but the mutant caspase-8

Table 1 Phenotype of kindred that are genetically deficient for caspase-8

Subject*	Patient 1	Patient 2	Mother 66	Sister 66
Total lymphocytes (832–2,028)	1,510	2,362	1,456	1,677
CD4 % (32.6–58.9)	23.1	25.0	42.3	36.2
CD8 % (17.8–46.7)	49.6	46.2	30.7	32.3
CD4/CD8 ratio (0.75–3.31)	0.5	0.5	1.4	1.1
B lymphocytes (88–330)	361	570	326	354
Clinical symptoms				
Failure to thrive/short stature	+	+	–	–
Lymphadenopathy	+	+	–	–
Splenomegaly	+	+	–	–
Eczema	+	+	–	–
Reactive airway disease	+	+	–	–
HSV labialis	+	+	–	–
Pneumonia	+	+	–	–
Asthma	+	+	–	–
Chronic diarrhoea	–	+	–	–
Response to pneumococcal immunization	–	–	ND	+
Immunoglobulin concentrations (mg dl ^{–1})				
IgG (723–1,685)	857	544	ND	864
IgA (81–463)	112	56	ND	114
IgM (48–271)	52	32	ND	27
IgE (<180)	7	20	ND	28

*The normal range for each value is shown in parentheses. ND, not determined.

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did not (ref. 16 and Fig. 2d). In an apoptosis assay in which fusion to the CD8 ectodomain spontaneously drives the multimerization of caspase-8 (ref. 17), the wild-type construct (CD8-C8wt) induced cell death, whereas the mutant construct (CD8-C8mt) was inactive (Fig. 2e). Densitometry showed that the abundance of mutant caspase-8 protein in the homozygotes was less than 5% of the wild-type amount, indicating that the mutant protein is unlikely to be partially functional or have allosteric regulatory effects. Thus, the Arg248Trp mutation reduces the stability of the caspase-8 protein and renders it enzymatically inactive.

The association between the homozygous caspase-8 mutation and immunodeficiency led us to study lymphocyte activation responses. We found interleukin-2 (IL-2) production was markedly defective when PBLs from individuals homozygous for the mutant caspase-8 were stimulated through the T-cell receptor (TCR; Fig. 3a). Notably, the heterozygous family members had a partial defect in IL-2 release. IL-2 production was rescued by stimulation with phorbol myristate acetate (PMA) and ionomycin, which bypass proximal TCR signalling and induce second messengers directly (Fig. 3b). We also found diminished T-cell proliferation responses to phytohaemagglutinin (PHA) in the two homozygotes, whereas the heterozygotes showed minimal proliferative defects (Fig. 3c).

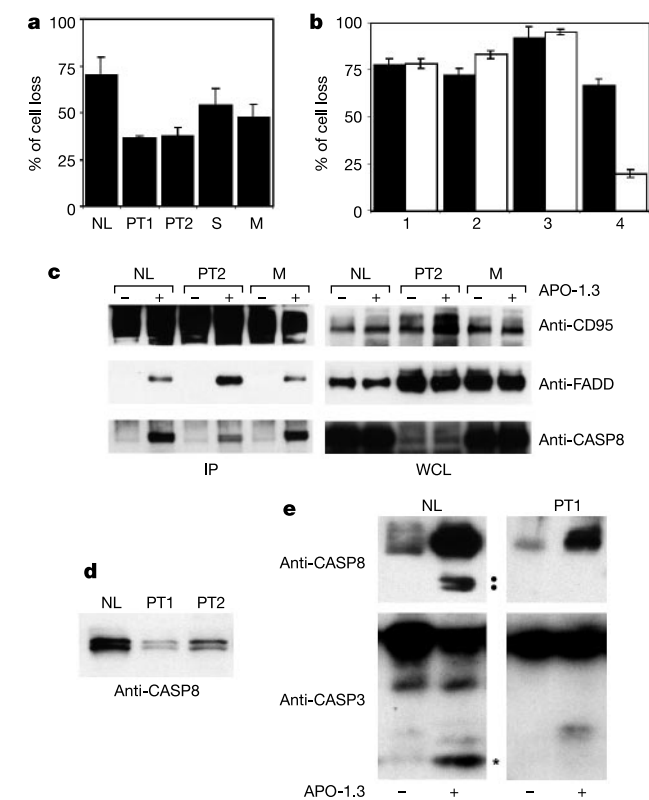


Figure 1 Defective apoptosis and DISC analysis in family 66. **a**, Activated PBLs from normal volunteers (NL), patient 1 (PT1), patient 2 (PT2), the sister (S) and mother (M) were stimulated with antibody to CD95 and analysed for cell loss. **b**, B cells transformed with the Epstein-Barr virus (EBV) were treated with staurosporine (1), etoposide (2), ceramide (3) or antibodies to CD95 (4) and analysed for cell loss. Filled bars represent normal control cells, open bars represent PT1 cells. **c**, DISC immunoprecipitates (IP) using antibodies to CD95 and whole-cell lysates (WCLs) from PBLs either treated (+) or untreated (–) with antibody to CD95 (APO-1.3) were blotted using antibody to CD95, FADD or caspase-8. **d**, Immunoblot showing amount of caspase-8 in PBL lysates. **e**, DISC immunoprecipitates from cells with or without anti-CD95 crosslinking were blotted with antibody to caspase-8 (top), and the corresponding lysates were blotted with antibody to caspase-3 (bottom). Cleavage products of caspase-8 and caspase-3 are marked by dots and an asterisk, respectively. Data are representative of four experiments.

CD4- and CD8-positive T-cell populations from patients 1 and 2 showed calcium defects, indicating that the defect in activation affected both subsets of T cells (Fig. 3d and data not shown).

We examined the influence of caspase-8 on surface activation markers after lymphocyte stimulation (Table 2). The basal surface expression of CD3, CD28 and CD95 was normal (data not shown); however, CD25 induction was markedly defective on both CD4- and CD8-positive T lymphocytes from patients 1 and 2. Induction defects were also observed for CD28, CD71, CD95, CD134, CD152 and MHC class II molecules, whereas induction of CD137 and downregulation of the TCR were not affected (Table 2 and data not shown). Cells from heterozygous family members also showed substantial defects in activation marker expression that were exacerbated by the caspase inhibitor zVAD-fmk (Table 2 and data

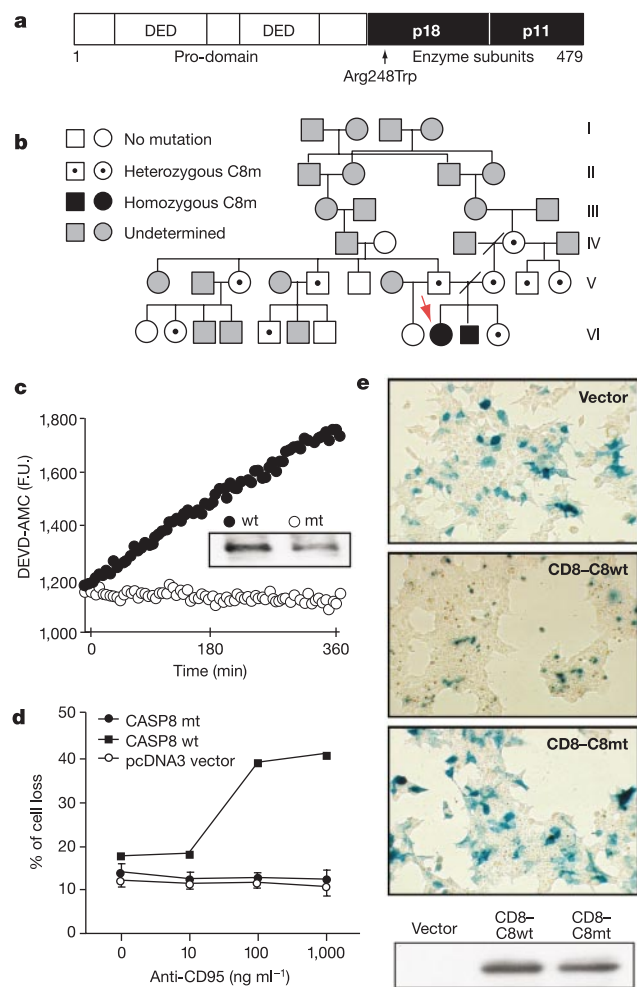


Figure 2 Characterization of an inherited caspase-8 mutation. **a**, Caspase-8 protein showing the death effector domains (DED) and the enzyme subunits (p18 and p11). The location and predicted amino acid substitution of the mutation are indicated by the arrow. **b**, Pedigree of family 66. Patient 1 is indicated by the red arrow. **c**, Measurement of DEVD-AMC cleavage using GST fusion proteins of wild-type (wt, filled circles) or Arg248Trp mutant (mt, open circles) caspase-8. Inset, immunoblot for caspase-8 shows that nearly equivalent amounts of the two GST-caspase-8 fusion proteins were used in the assay. F.U., arbitrary fluorescence units. **d**, Jurkat I9.2 cells were transfected with vector (open circles), wild-type caspase-8 (filled squares) or Arg248Trp mutant caspase-8 (filled circles), treated with antibody to CD95, and assessed for cell loss. **e**, 293T cell lines were transfected with vector (top), or with CD8 fusions of wild-type (middle) or mutant Arg248Trp (bottom) caspase-8 along with a β -galactosidase expression vector. The immunoblot of transfected cell lysates with antibody to caspase-8 shows similar expression of caspase-8 protein. Data are representative of three experiments.

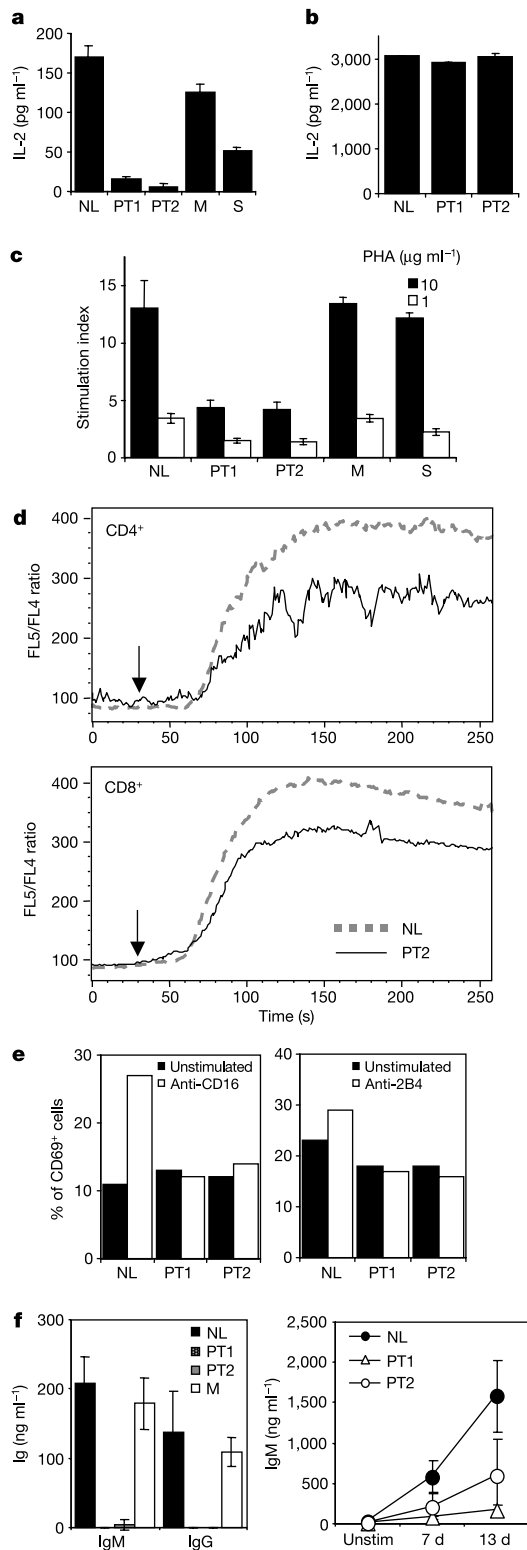


Figure 3 Defective immune cell responses in homozygous caspase-8 mutants. **a**, IL-2 release after stimulation with antibodies to CD3 and CD28 (10 $\mu\text{g ml}^{-1}$ each) for family members abbreviated as in Fig. 1. **b**, IL-2 release after stimulation with PMA and ionomycin. **c**, Stimulation index measured by thymidine incorporation after treatment with PHA. **d**, Calcium flux in CD4⁺ (top) or CD8⁺ (bottom) cells after TCR and CD28 stimulation. **e**, CD69 expression in purified NK cells after stimulation with antibodies to CD16 (left) or to 2B4 (right). **f**, Production of immunoglobulins by PBLs that were unstimulated (unstim) or stimulated with pokeweed mitogen (PWM; left) or by purified B cells that were stimulated with SAC and IL-2 for the indicated number of days (right). Data are representative of four experiments.

not shown). By contrast, control experiments showed that lymphocytes from individuals with conventional ALPS and CD95 mutations did not have defective induction of surface activation molecules (data not shown). These results show that caspase-8 has a pivotal role in a proximal signalling event that is essential for a selected subset of TCR-induced responses.

A prominent feature of human homozygous caspase-8 deficiency is severe infection by mucocutaneous HSV. Because defects in natural killer (NK) cells predispose to herpes virus infections, we examined signalling by the CD16 and 2B4 pathways in purified NK cells^{18–20}. Activation of NK cells manifested by CD69 expression was induced by stimulating the cells with antibodies to CD16 or 2B4 in the control samples, but not in samples from patients 1 or 2 (Fig. 3e). Defective NK cell activation by 2B4 could be reconstituted in normal controls by pretreatment with zVAD-fmk (data not shown). The variable decreases in serum immunoglobulin concentrations in these individuals also prompted us to test their B-cell function. We

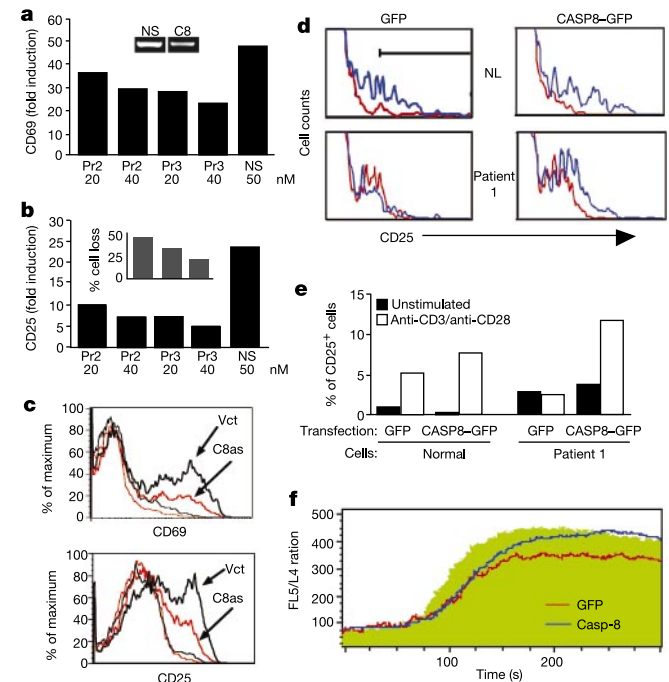


Figure 4 Caspase-8 is required for lymphocyte activation. **a**, Induction of the surface expression of CD69. Resting PBLs were transfected for 16 h with RNAi primers that were either nonspecific (NS) or specific for caspase-8 (primer pair 2 or 3), and were then stimulated with TCR and CD28 for 18 h. Inset shows that less caspase-8 mRNA is produced by RT-PCR with primer pair 3 (C8) than with the nonspecific primers (NS). **b**, Induction of the surface expression of CD25 analysed as in **a**. Inset shows a reduced percentage of cell loss after anti-CD95 antibody (500 ng ml⁻¹) stimulation of HeLa cells transfected with primer pair 2 (2) or primer pair 3 (3), as compared with nonspecific primers (1). **c**, Flow cytometry of PBLs co-transfected with 2 μg of pEGFP and 5 μg of vector (Vct, black) or a caspase-8 antisense construct (C8as, red) and cultured for 72 h before treatment with control medium (thin curves) or TCR and CD28 stimulation (1 $\mu\text{g ml}^{-1}$ each, thick curves) for 6–24 h. Expression of CD69 at 6 h (top) or CD25 at 24 h (bottom) is shown. Protein blots show less expression of caspase-8 in samples transfected with C8as as compared with controls; the results in **a–c** were reproduced with eight random blood donors. **d**, Flow cytometry analysis of the surface expression of CD25 by normal (NL) and patient 1 PBLs transfected with GFP control (left) or wild-type caspase-8–GFP fusion protein (right) and either stimulated with antibodies to CD3 and CD28 (blue line) or left unstimulated (red line). Black line indicates the positive gate used for quantification. **e**, Representation of the results shown in **d**. **f**, Calcium flux after stimulation with antibodies to CD3 and CD28 in PBLs from normal controls (green area) or from patient 1, transduced with either GFP control (red line) or caspase-8–GFP retrovirus (blue line). Data are representative of three experiments.

found that PBLs stimulated with pokeweed mitogen (PWM), a T-cell-dependent mitogen, showed markedly decreased production of immunoglobulin- μ (IgM) and immunoglobulin- γ (IgG) in patients 1 and 2, whereas the mother showed normal production of immunoglobulins (Fig. 3f). IgM release was also impaired when purified B cells from patients 1 and 2 were stimulated with the T-cell-independent mitogen *Staphylococcus aureus* Cowan I (SAC) plus exogenous IL-2 (Fig. 3f). Thus, immunodeficiency and the resultant opportunistic infections associated with homozygous caspase-8 mutation reflect pleiotropic defects that involve the functions of T, NK and B cells.

We validated the association of caspase-8 deficiency with defective lymphocyte activation by two approaches. First, we inactivated caspase-8 in normal human peripheral blood lymphocytes with RNA interference (RNAi) and antisense techniques. Using an electroporation apparatus (Nucleofector System, Amaxa) for efficient gene transfection of resting human PBLs, we introduced two different duplex RNAi ribonucleotides specific for caspase-8 and then stimulated the cells through the TCR complex²¹. Induction of CD69 and CD25 showed that RNAi primer pairs 2 and 3 substantially reduced the response to TCR stimulation as compared with nonspecific primers (Fig. 4a, b). Controls showed that the specific primer pairs reduced caspase-8 messenger RNA and decreased CD95-induced apoptosis (Fig. 4a, b, insets). In addition, transfection of caspase-8 antisense constructs impaired CD69 and CD25 induction as compared with vector controls, further showing that interfering with expression of the caspase-8 gene blocks activation responses (Fig. 4c).

Second, we transfected lymphocytes from patient 1 with an expression construct that encoded either a wild-type fusion protein of caspase-8 and green fluorescent protein (GFP) or GFP alone. Cells transfected with the GFP control did not show upregulation of CD25 expression after stimulation, but cells transfected with the wild-type caspase-8–GFP construct showed a threefold or greater induction of CD25 expression, comparable to normal controls (Fig. 4d, e). Introduction of a functional caspase-8 molecule by lentiviral gene transduction also rescued the impaired calcium response to TCR stimulation in cells from patient 1 (ref. 22 and Fig. 4f). We also found that the caspase-8-deficient I9.2 Jurkat line manifested defects in activation responses that could be corrected by retroviral expression of the wild-type caspase-8 gene (Supplementary Information). Together, these findings show that caspase-8 is necessary for proximal signalling events in lymphocytes.

Our analysis of individuals with inherited caspase-8 deficiency has shown that caspase-8 has a broad role in the activation of T, B and NK cells in addition to its function in conveying signals from death receptors to apoptosis effector mechanisms^{12,13}. Our results also explain how chemical caspase inhibitors interfere with T-cell proliferation^{12–14}. Caspase-8 probably functions in lymphocyte activation independently of CD95, because individuals with type I ALPS and defects in CD95 do not have immunodeficiency, which

makes it unlikely that this ligand–receptor pair is involved²³. The immunodeficiency associated with defective caspase-8 also contrasts with the autoimmune phenotype of individuals with caspase-10 deficiency, despite the structural homology of these two enzymes¹¹. Because mice that are deficient in caspase-8 show embryonic lethality, humans that lack caspase-8 function might not be expected to survive. Postnatal survival in humans may be due to the function of caspase-10, the closest paralogue of caspase-8 in humans. Caspase-10 has no known orthologue in mice but may compensate for caspase-8 (refs 3, 4). The phenotypes associated with caspase-8 and caspase-10 mutations in humans are based on limited genetic data, and studying more individuals with these disorders may define better the different consequences of these mutations. The defects in activation of T, NK and B cells suggest that there may be a common locus of action for caspase-8 in several lymphocyte signalling pathways that will be interesting to uncover. Caspase-8 may be a potentially useful target for a new class of anti-inflammatory or immunosuppressive therapeutics and there may be non-apoptotic signalling roles for caspases in cellular responses of non-immune organ systems. Hence, caspase-8 establishes a close link between the biochemical pathways of cellular activation and apoptosis that may shed light on the homeostatic regulation of these seemingly conflicting processes. □

Methods

Cellular preparations and analysis

Individuals were studied under NIH-approved ALPS research protocols with informed consent. Wild-type (IA3) and caspase-8-deficient (I9.2) Jurkat cells were gifts from J. Blenis. The 293T cell line was obtained from ATCC. Peripheral blood T lymphocytes were separated on Ficoll gradients and stimulated first with 5 $\mu\text{g ml}^{-1}$ of PHA (Sigma) for 3 d and then with 100 IU ml^{-1} IL-2. Apoptosis assays used 10–1,000 ng ml^{-1} antibodies to CD95 (APO-1.3, Kamiya), 0.1 $\mu\text{g ml}^{-1}$ staurosporine, 10 $\mu\text{g ml}^{-1}$ etoposide or 10 μM ceramide as described^{5,24}. We purified B cells using fluorescein isothiocyanate (FITC)-labelled antibodies to CD19, followed by positive selection with anti-FITC Microbeads (MACS). We prepared NK cells by negative selection using the NK Cell Isolation Kit (MACS). Immunoprecipitation and western blot analyses were carried out using antibodies to caspase-3 and FADD (Transduction Laboratories), CD95 (Kamiya) and caspase-8 (a gift from M. Peter).

Sequence analysis, plasmids and mutagenesis

We purified RNA using Trizol (Gibco-BRL) and carried out polymerase chain reaction with reverse transcription (RT–PCR) using the OneStep RT–PCR kit (Qiagen). Sequence analysis was done by dye primer chemistry (Amersham) on an ABI 377 automated sequencer and also by Cleveland Genomics. Bacterially expressed GST fusion proteins, full-length caspase-8 fused to GFP at the carboxy terminus for transfection studies, and CD8–caspase-8 constructs were prepared as described^{11,17}. We generated mutations using the QuickChange Site-Directed Mutagenesis kit (Stratagene).

Biochemical and functional assays

Purification of GST fusion proteins and caspase assays using DEVD-AMC have been described¹¹. We transfected I9.2 cells with various constructs and a GFP control vector at a molar ratio of 3:1. After 18 h, cells were treated with antibody to CD95 and assayed for apoptosis. Semiconfluent 293T cells or HeLa cells (RNAi) were transfected using Eugene 6 (Roche) according to the manufacturer's instructions with 2 μg of caspase-8 constructs and 1 μg of a 3LacZ construct. Cells were then fixed and stained for β -galactosidase as described¹⁷. To transfect resting PBLs, we mixed 5 μg of DNA containing the GFP fusion proteins with 100 μl of the human T-cell Nucleofector solution and then used this to resuspend 5×10^6 PBLs that had been freshly isolated using Ficoll. The PBL suspension was immediately electroporated by a Nucleofector instrument (Amaxa Biosystems). The transfected cells were added to 1 ml of complete RPMI-1640 medium for 24 h before being exposed to plate-bound anti-CD3 and anti-CD28 antibodies (each at 10 $\mu\text{g ml}^{-1}$, Pharmingen) for 24 h.

For RNAi analysis, primer pair 2 (5'-AATCACAGACTTTGGACAAAG-3' and 5'-AACTTTGTCCAAAGTCTGTGA-3', starting at nucleotide 653 from caspase-8 ATG), primer pair 3 (5'-AACTACCAGAAAGGTATACCT-3' and 5'-AAAGGTATACCTTTC TGCTAG-3', starting at nucleotide 1,090 from caspase-8 ATG) and nonspecific (NS) primers (5'-AACACGTAGCAGCTCGGATCG-3' and 5'-AACGATCCGAGCTGCTAC GTG-3') were used in the Silencer siRNA construction kit (Ambion). The caspase-8 primers used in RT–PCR were FLICE Fr (5'-CCTTGGGAATATTGAGATTATATTCTCC-3') and FLICE Rev (5'-ATAGCACCATCAATCAGAGGGAAGCAAG-3'). We generated the antisense construct by inserting the 498-base-pair *EcoRI/XmaI* caspase-8 cDNA fragment including the translation start site into a pEGFP-C1 vector (Clontech). The expression of CD25 and CD69 was determined by staining the surface of cells with the corresponding phycoerythrin-conjugated antibodies (Pharmingen). We analysed the data after gating on live GFP-positive cells.

Table 2 Fluorescence of surface markers after CD3/CD28 antibody stimulation

Surface markers	Normal (+/+)	Patient 1 (–/–)	Patient 2 (–/–)	Normal + 50 μM zVAD-fmk
CD25/CD4	514	42	97	18
CD25/CD8	417	23	112	10
CD28	510	178	316	170
CD71	204	30	64	16
CD95	141	39	60	54
CD134	62	22	50	13
CD137	20	17	44	6
CD152	26	12	16	13
MHC class II molecules	120	45	47	23

Geometric mean fluorescence of surface activation molecules after stimulation with antibodies to CD3 and CD28. The geometric mean fluorescence of each marker was calculated from the CD3-positive populations (or CD4- and CD8-positive populations for CD25). Unstimulated cells had similar values of geometric mean fluorescence. Data are representative of ten experiments.

Stimulation, cytokine measurements and proliferation assays

Resting PBLs were stimulated for typically 24 h with $1 \mu\text{g ml}^{-1}$ anti-CD3 and $5 \mu\text{g ml}^{-1}$ anti-CD28 antibodies (Pharmingen) or with $5 \mu\text{g ml}^{-1}$ PHA. We stimulated NK cells with $50 \mu\text{g ml}^{-1}$ of either plate-bound anti-CD16 (3G8) or anti-2B4 (C1.7) antibodies (Pharmingen) for 24 h. B lymphocytes were stimulated with either $10 \mu\text{g ml}^{-1}$ pokeweed mitogen (Sigma) or 10% (v/v) of SAC cells (Calbiochem) and 200 IU ml^{-1} of IL-2. Surface molecules of interest were detected using phycoerythrin (PE)- and FITC-labelled antibodies (Pharmingen) by standard procedures, and analysed on a FACScan flow cytometer (Becton-Dickinson) using CellQuest (Applied Biosystems) and FlowJo (TreeStar) software. Concentrations of immunoglobulin in the supernatants of cells at days 7 and 13 were measured by R. Hornung at the Immunological Monitoring Laboratory of the National Cancer Institute. For stimulations with PMA and ionomycin, 10 ng ml^{-1} and $0.5 \mu\text{g ml}^{-1}$ were used, respectively. Where indicated, cells were pretreated for 36 h with $50 \mu\text{M}$ zVAD-fmk (Enzyme Systems Products). We measured IL-2 after 48 h of stimulation using the Quantikine Immunoassay Kit (R&D Systems). In proliferation assays, cells stimulated for 48 h with PHA and anti-CD28 antibody were pulsed with tritiated thymidine for 24 h and assessed by scintillation counting.

Luciferase and calcium flux assays

Jurkat T cells were transfected with $10 \mu\text{g}$ of an IL-2–Luc construct and $0.2 \mu\text{g}$ of a renilla construct as described²⁵, stimulated with $20 \mu\text{g ml}^{-1}$ of plate-bound anti-CD3 and anti-CD28 antibodies for 24 h, and analysed with the Luciferase Assay System (Promega). To measure intracellular calcium, we loaded PBLs with 3 mM Indo-1 acetoxymethyl ester (Indo-1, Molecular Probes), incubated them at 37°C and then stimulated them with $1 \mu\text{g ml}^{-1}$ of anti-CD3 and $5 \mu\text{g ml}^{-1}$ of anti-CD28 antibodies for about 6 min. The Indo-1 ratio of 395 nm/500 nm fluorescence emission was calculated by flow cytometry.

Retroviral infections

Lentiviral transduction of PBLs used plasmids that contained, in order, the HIV long terminal repeat (LTR), the caspase-8 coding sequence, an internal ribosome entry site (IRES) and GFP (HIV-LTR–caspase-8–IRES–GFP) or HIV-LTR–IRES–GFP, together with a packaging vector ($\Delta 8.2$) and a CMV–VSV-G construct (a gift from F. Candotti)²² or the kat system²⁶. We transfected the lentiviral vectors into 293T cells to generate viral supernatants, which we then concentrated by spinning.

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A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*

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Localized attack by a necrotizing pathogen induces systemic acquired resistance (SAR) to subsequent attack by a broad range of normally virulent pathogens. Salicylic acid accumulation is required for activation of local defenses, such as pathogenesis-related protein accumulation, at the initial site of attack, and for subsequent expression of SAR upon secondary, distant challenge^{1,2}. Although salicylic acid moves through the plant, it is apparently not an essential mobile signal². We screened *Agrobacterium tumefaciens* transfer DNA (tDNA) tagged lines of *Arabidopsis thaliana* for mutants specifically compromised in SAR. Here we show that *Defective in induced resistance 1-1* (*dir1-1*) exhibits wild-type local resistance to avirulent and virulent *Pseudomonas syringae*, but that pathogenesis-related gene expression is abolished in uninoculated distant leaves and *dir1-1* fails to develop SAR to virulent *Pseudomonas* or *Peronospora parasitica*. Petiole exudate experiments indicate that *dir1-1* is defective in the production or transmission from the inoculated leaf of an essential mobile signal. *DIR1* encodes a putative apoplastic lipid transfer protein and we propose that

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DIR1 interacts with a lipid-derived molecule to promote long distance signalling.

Genetic screens for mutants with impaired basal resistance to initial attack by virulent pathogens or an impaired response to exogenous salicylic acid have identified signalling genes involved in both local and distant activation of defence mechanisms³. To identify genes involved in the entire SAR pathway, including long-distance signalling, the main feature of SAR, we screened a collection of tDNA tagged *A. thaliana* ecotype Wassilewskija (Ws) lines⁴ for mutants that fail to develop SAR after inoculation with avirulent *P. syringae* pv *tomato* (*Pst*).



Figure 1 Characterization of *dir1-1*. **a**, Symptoms in uninduced (MV) and induced (AV) Ws and *dir1-1* plants 3 days post-challenge inoculation with virulent *Pst*. **b**, Ws and *dir1-1* were subjected to MV, MA and AV treatments. Treatments consist of inoculation in one leaf (first letter), followed 2 days later with the second inoculation in other leaves (second letter). M, mock inoculation; A, inoculation with avirulent *Pst* (first inoculation at 10⁶ CFU ml⁻¹ or second inoculation at 10⁵ CFU ml⁻¹; V, inoculation with virulent *Pst* at 10⁵ CFU ml⁻¹. Bacterial growth in plants was monitored after the second inoculation. A significant difference was observed between Ws MV and AV treatments (*t*-test, *P* < 0.05). This experiment was repeated with similar results at least four times.

Plants were induced by inoculating one leaf with avirulent *Pst* (*PstavrRpt2*); after 2 days, three other leaves were challenge-inoculated with virulent *Pst*. Of 11,000 tDNA lines screened only one, *dir1-1*, was specifically compromised in SAR. The SAR-defective phenotype, as manifested by bacterial growth in plants equivalent to that observed following challenge of uninduced control plants with virulent *Pst*, segregated 3:1 (chi square, *P* > 0.9, *P* > 0.8, in independent experiments). These results indicated that the original line was heterozygous and that the *dir1-1* mutation was dominant to *DIR1* or that the wild-type allele was haplo-insufficient. These conclusions were confirmed by backcrossing three different *dir1-1* plants to wild-type Ws.

The *dir1-1* line contained more than one tDNA insertion. A SAR-defective kanamycin-resistant individual was back-crossed to Ws to remove unlinked tDNA inserts. One hundred F₂ families were tested for kanamycin resistance and SAR competence. A *dir1-1* SAR-defective line was isolated and F₃ plants were monitored for cosegregation of kanamycin resistance and the SAR defect. This *dir1-1* line was used in subsequent studies and later shown to be homozygous for the *dir1-1* allele in polymerase chain reaction (PCR) experiments using *DIR1* and *dir1-1* gene-specific primers. Experiments with homozygous or heterozygous *dir1-1* lines confirmed that *DIR1* was either recessive to *dir1-1* or haplo-insufficient with respect to the SAR phenotype, as measured by bacterial growth in plants.

Challenge inoculation of *dir1-1* homozygotes with virulent *Pst* 3

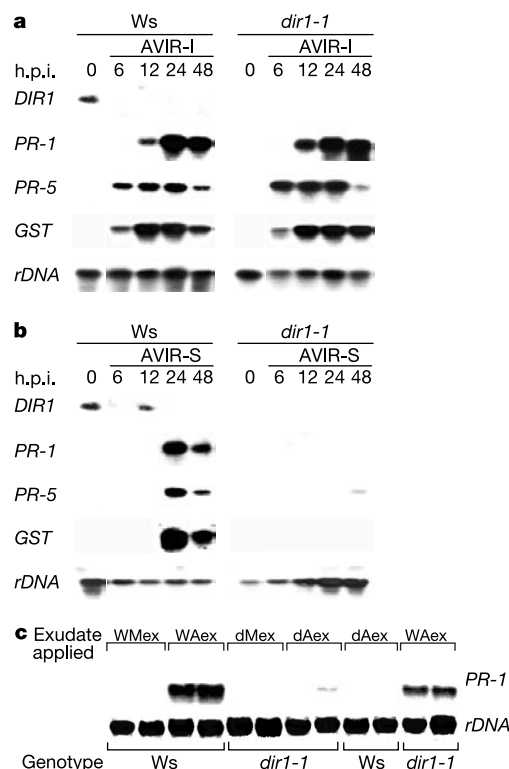


Figure 2 Defence gene expression in Ws and *dir1-1*. Leaves were collected at 0, 6, 12, 24 and 48 hours post inoculation (h.p.i.) from plants inoculated (I) with *Psm-avrRpm1*, 10⁷ CFU ml⁻¹ (**a**) and from uninoculated or systemic (S) leaves (**b**). RNA gel blots were hybridized with *PR-1*, *PR-5*, *GST* and *rDNA* gene probes. Two *PstavrRpt2/PR-1* expression experiments displayed similar results. **c**, Petiole exudates were infiltrated into naïve Ws and *dir1-1* plants (labelled Genotype). *PR-1* and *rDNA* gene expression was monitored by RNA gel blot analysis 1 day post exudate infiltration; duplicate samples are shown. Exudates were WMex (from Ws mock-inoculated leaves), WAex (from Ws leaves inoculated with avirulent *Pst*, 10⁶ CFU ml⁻¹), dMex (from *dir1-1* mock-inoculated leaves) and dAex (from *dir1-1* leaves inoculated with avirulent *Pst*). This experiment was repeated twice with similar results.

days after local inoculation with avirulent *Pst* resulted in chlorosis and vigorous bacterial growth similar to that in uninduced wild-type plants (Fig. 1a, b). Fivefold greater bacterial growth (Student's *t*-test, $P < 0.002$) was observed in systemic leaves of *PstavrRpt2*-induced *dir1-1* plants challenged with virulent *Pst* (AV treatment; see legend to Fig. 1 for description) compared to equivalent leaves in wild-type plants (Fig. 1b). In contrast, there was no significant difference between the growth of virulent *Pst* in uninduced, naïve *dir1-1* plants (MV treatment) compared to equivalent wild-type plants 1 day (data not shown) or 3 days post inoculation (d.p.i., Fig. 1b). Moreover, naïve *dir1-1* plants restricted the growth of *PstavrRpt2* (MA treatment) to a similar extent as wild-type 1 d.p.i. (data not shown) or 3 d.p.i. (Fig. 1b). Therefore, although the mutant is impaired in *PstavrRpt2*-induced SAR, naïve *dir1-1* plants respond as wild-type to local inoculation with avirulent or virulent *Pst* and likewise produce an oxidative burst⁵ and a visible hypersensitive response (HR) (data not shown). In addition, SAR was not established in *dir1-1* using *PstavrRpm1* as the inducer (data not shown). Therefore the SAR defect in *dir1-1* plants is not specific to the inducing gene-for-gene interaction. *PstavrRpt2*-induced SAR to the unrelated oomycete pathogen, *Peronospora parasitica*, was also abolished in the *dir1-1* mutant (see Supplementary Information).

Local accumulation of *PR-1*, *PR-5* and glutathione-S-transferase (*GST*) transcripts in leaves inoculated with *PstavrRpm1* was similar in Ws and *dir1-1* plants, but was greatly reduced in the distant uninoculated leaves of *dir1-1* plants (Fig. 2a, b; Supplementary Information Fig. 1). Similar results were obtained with *PstavrRpt2* as the inducer (data not shown), providing further evidence that gene-for-gene local resistance is unimpaired in *dir1-1*. However, loss of *PR-1* expression in distant leaves indicates that *dir1-1* is defective in long-distance signalling.

DIR1 might function in production of the mobile signal, its transmission from the inoculated leaf or its perception in distant leaves. To distinguish between these sites of action we collected petiole exudates (enriched for phloem sap) from leaves that had been induced by inoculation with avirulent *Pst* and assayed the exudates for *PR-1* gene-inducing activity after infiltration into leaves of healthy, untreated plants (Fig. 2c). Exudates from Ws leaves induced by inoculation with avirulent *Pst* elicited *PR-1*

expression in leaves of healthy, untreated wild-type Ws plants, whereas exudates collected from mock-inoculated Ws leaves had little activity. Moreover, exudates from induced wild-type leaves elicited *PR-1* expression in recipient leaves of healthy, untreated *dir1-1* plants. In contrast, exudates from *PstavrRpt2*-induced *dir1-1* leaves elicited little *PR-1* gene expression in recipient leaves of either wild-type Ws or *dir1-1* plants. Thus we conclude that *DIR1* functions in the production of an essential mobile signal or its transmission from the emitting leaf, rather than in the perception or transduction of the signal in recipient leaves.

Salicylic acid is an essential secondary signal for both SAR and local resistance and 2,6-dichloroisonicotinic acid (INA) mimics salicylic acid functions⁶. Free salicylic acid levels were similar in Ws and *dir1-1* plants throughout the SAR response: in the locally inoculated leaf (induction), in distant uninoculated leaves (establishment) and after challenge inoculation in distant leaves (manifestation); see Supplementary Information and Table 1. Thus *DIR1* is not required for the accumulation of salicylic acid either locally or systemically. However, spraying with INA induced resistance in both *dir1-1* and the wild type, with 19/19 and 10/11 resistant plants respectively, compared to 0/12 for water-sprayed control plants. Therefore, *DIR1* is not required for salicylic acid accumulation, but salicylic acid may contribute to the pathway downstream of *DIR1*.

Genomic DNA blot hybridization showed that a full-length tDNA plus an inverted partial tDNA were inserted in the *dir1-1* line (Fig. 3 a, b). Thermal asymmetric interlaced (TAIL)-PCR⁷ was used to obtain the corresponding wild-type genomic DNA (Gen-

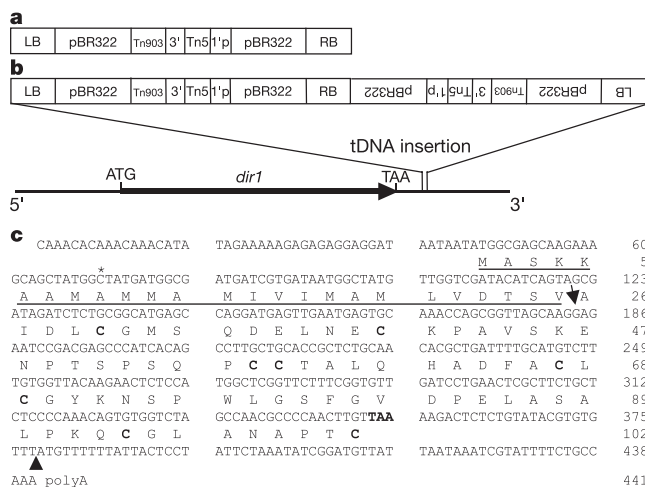


Figure 3 Structure of the tDNA insertion in the *dir1-1* allele. **a**, Structure of the 3850:1003 tDNA^a. **b**, The tDNA insertion in the *dir1-1* allele. **c**, Sequence analysis of *DIR1*. The putative signal peptide is underlined, and the signal sequence cleavage site is indicated by an arrow. The translational stop codon, TAA, is in bold. The tDNA insertion in the 3' untranslated region in the *dir1-1* line is indicated by a black triangle. The eight conserved cysteine residues are shown in bold. Position 71 (asterisk) shows an amino acid polymorphism between Ws (Ala) and Col-0 (Val) *Arabidopsis* ecotypes.

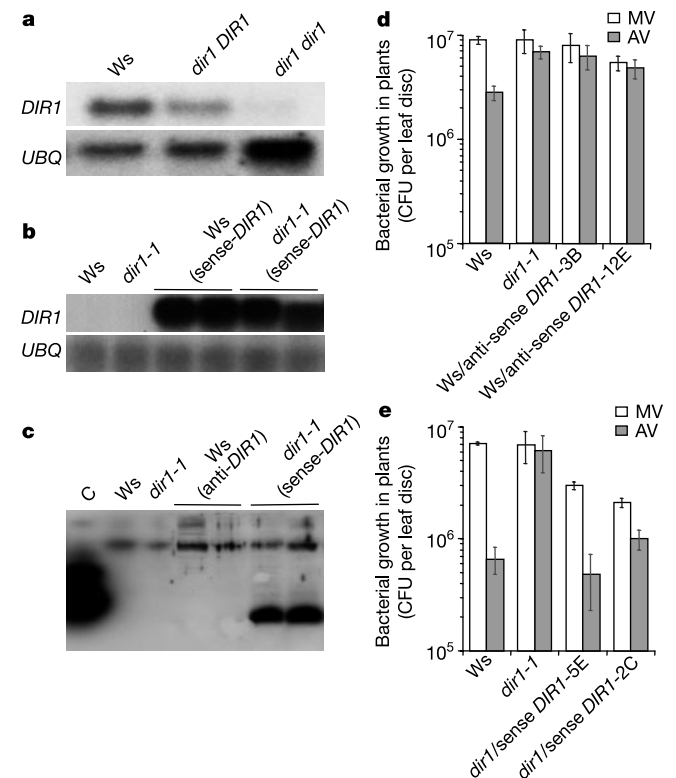


Figure 4 Complementation of *dir1-1*. **a**, Basal levels of *DIR1* transcripts. RNA gel blot analysis of *DIR1* gene expression in Ws, heterozygous and homozygous *dir1-1*. **b**, RNA gel blot analysis of *DIR1* transcript levels in untreated leaves from Ws, *dir1-1*, Ws(sense-*DIR1*) and *dir1-1* (sense-*DIR1*). **c**, Protein gel blot analysis of *DIR1*/LTP levels using an LTP polyclonal antibody. C, control: purified recombinant *DIR1*. **d**, Loss of SAR in anti-sense transgenic lines. Significant difference observed in Ws (MV versus AV), $P < 0.001$; no significant difference in *dir1-1* (MV versus AV) or anti-sense lines (MV versus AV), $P > 0.3$. **e**, SAR in over-expression transgenic lines. Significant differences observed between Ws, MV and AV, $P < 0.04$; and between over-expression lines, MV and AV, $P < 0.04$. Experiments in **d** and **e** were repeated three times each with similar results.

bank accession number AF342726). The cloned DNA was contiguous in the wild-type genome and interrupted by the tDNA in the *dir1-1* line. Sequence analysis of PCR products from the *dir1-1* tDNA flanking DNA revealed an additional 80 bp compared to the wild-type sequence (Fig. 3b). The tDNA inserted in the 3' untranslated region of *DIR1*, 25 base pairs (bp) downstream of the stop codon (Fig. 3b).

DIR1 sequences hybridized to a single, low-abundance transcript of about 0.5 kilobases (kb) in total RNA preparations from healthy wild-type plants. This transcript was barely detectable in *dir1-1/dir1-1* plants and present at intermediate levels in the heterozygote (Fig. 4a). Inoculation of wild-type plants with avirulent *P. syringae* pv. *maculicola*, (*Psm*)*avrRpm1*, caused a reduction in *DIR1* transcript levels in the inoculated leaf, but had some (Fig. 2b) or little (Fig. 1 of the Supplementary Information) effect on *DIR1* transcript levels in distant, uninoculated leaves. *DIR1* genomic sequences were used as hybridization probes to screen an *Arabidopsis* CD4-7 complementary DNA library⁸ and two full-length cDNAs were recovered. *DIR1* contains a 306-nucleotide open reading frame with no intron, encoding a protein of 102 amino acids with a predicted relative molecular mass of 10,600 (Fig. 3c). *DIR1* shares a region of 54 nucleotides with a *Phaseolus vulgaris* nonspecific lipid transfer-like protein (LTP) and displays 53% sequence similarity throughout the protein. The predicted *DIR1* protein contains a hydrophobic amino-terminal signal sequence and the eight cysteine residues conserved in all LTPs⁹, suggesting that *DIR1* encodes an apoplastic LTP.

DIR1 was mapped using recombinant inbred lines (Nottingham *Arabidopsis* Stock Centre) with confirmation by analysis of the *Arabidopsis* Genome Initiative sequence map (clone MJE7). *DIR1* mapped to chromosome 5, position 84.5 cM, between markers ET01 (83.5 cM) and EMB15 (85.0 cM) near *LEAFY* (*LFY*, locus AT5G61850, 82.0 cM).

Because *DIR1* is either haplo-insufficient or recessive to *dir1-1*, we made antisense *DIR1* lines in the wild-type background and 35S::*DIR1* sense expression lines in both *dir1-1* and wild-type backgrounds. *DIR1* transcripts accumulated abundantly in the leaves of the overexpression lines (Fig. 4b). A *DIR1* LTP polyclonal antibody was generated (see Supplementary Information) and used in protein gel blot analyses. Very little *DIR1* protein was detected in the leaves of either the wild-type, *dir1-1* mutant or the 35S::antisense *DIR1* lines; however, a strong signal was detected in leaves of the 35S::*DIR1* overexpression lines (Fig. 4c).

In functional complementation tests, Ws/anti-sense *DIR1* lines 3B and 12E allowed vigorous bacterial growth in plants after challenge inoculation of distant leaves with virulent *Pst* 3 days after local inoculation with *Pst*avrRpt2 (AV) (Fig. 4d). Bacterial growth was not significantly different from that observed in uninduced antisense plants (MV) ($P > 0.4$) and hence these antisense lines were, like the internal *dir1-1* controls, SAR defective ($P > 0.4$). In a second experiment (Fig. 4e), wild-type Ws plants displayed a more robust SAR response with an 11-fold reduction in bacterial growth after *Pst* challenge of plants locally induced with *Pst*avrRpt2 (AV), compared to equivalent uninduced control plants (MV) ($P < 0.03$). The *dir1-1* lines 5E and 2C- over-expressed wild-type *DIR1* and limited growth of *Pst* in plants when induced by local inoculation with avirulent *Pst* (AV) compared to uninduced controls (MV) (six fold, t -test $p < 0.03$ and two fold, $P < 0.04$, respectively). *DIR1* overexpression has little or no effect on resistance of naïve plants to virulent *Pst*, with only 1 (Fig. 4e) of 6 (data not shown) experiments showing a small, but significant effect on growth. However, overexpression of the *DIR1* protein complemented the *dir1-1* SAR defect, suggesting that *DIR1* is haplo-insufficient rather than recessive to *dir1-1*.

The *dir1-1* mutant is defective for SAR to both *Pst* and *P. parasitica*, but exhibits a wild-type gene-for-gene response (oxidative burst, accumulation of salicylic acid and bacterial growth in

plants) that would normally induce SAR and likewise, basal resistance to *Pst* is not compromised in *dir1-1*, unlike in defence mutants such as *npr1*, *pad4* and *eds1* (refs 10–13). The loss of PR-1 expression in distant leaves in *dir1-1* implies that *DIR1* is required for long-distance signalling during SAR and petiole exudate experiments indicate that *dir1-1* is compromised in the generation of an essential mobile signal or its transmission from the induced leaf rather than its perception or transduction in distant leaves.

In contrast to *npr1* (ref. 11), *dir1-1* is rescued by application of INA, which acts downstream in the pathway for the production of pathogenesis-related proteins⁶. In addition, *dir1-1* is unimpaired in local responses to infection with *Pst*. Together these data suggest that the *dir1-1* defect lies upstream of NPR1 function in distant leaves. We note that *dir1-1* still accumulates salicylic acid during the establishment and manifestation stages of SAR, whereas PR-1 gene expression and SAR are lost. Salicylic acid alone is not particularly potent as an inducer of defence mechanisms, but at physiological levels acts in an agonist-dependent manner to amplify or potentiate inducible defence mechanisms and the gene-for-gene HR^{14,15}. Although salicylic acid accumulates to wild-type levels in *dir1-1*, an essential signal from the induced leaf seems to be absent. It is also possible that salicylic acid accumulation observed in distant leaves and upon pathogen challenge in *dir1-1* is not associated with SAR, but rather with basal defense to *Pseudomonas*^{13,16,17}.

The *Arabidopsis* genome has at least 15 LTP genes and the *DIR1* non-specific LTP displays 30 to 75% similarity to members of this family¹⁸. LTPs bind to and transfer phospholipids between membranes *in vitro*, but there is little evidence for *in vivo* lipid transfer in plants or animals^{9,19}. Structural studies predict that plant LTPs contain an internal hydrophobic pocket that is large enough to accommodate a fatty acid or lysophospholipid²⁰. Plant nsLTPs contain signal peptides, some of which target them to the cell wall^{21,22}. LTPs are associated with diverse plant functions and some are upregulated in response to infection and exhibit antimicrobial activity⁹.

A role for *DIR1*/LTP in disease resistance signalling is consistent with the observation that some mammalian LTPs act as lipid sensors or are involved in phospholipase-C-linked signal transduction¹⁹. While the *DIR1*/LTP might in principle itself be a mobile signal, that is unlikely because overexpression of *DIR1* is not sufficient to induce SAR. Pathogen exposure is still required, implying that either *DIR1* activity or localization is regulated in some way, or that *DIR1* functions in cooperation with the mobile signal.

Lipid molecules such as oxylipins (jasmonic acid), phosphatidic acid and N-acyl ethanolamines are synthesized or released from membranes upon pathogen or insect attack. Some act as second messengers in plant defence signalling^{23–25}. In addition, *Arabidopsis* *PAD4* and *EDS1* (refs 26, 27) encode putative lipases and may initiate release of lipid-derived mobile signals. *DIR1*/LTP could be either a co-signal or act as a translocator for release of the mobile signal into the vascular system and/or chaperone the signal through the plant. Future studies will be directed at identifying possible bioactive ligands of the *DIR1*/LTP and tracking *DIR1* movement in plants.

Note added in proof: LTPs show some structural similarities to small cysteine-rich proteins secreted by *Phytophthora*. These elicitors activate disease resistance responses in some host species and can move systematically through the plant. Sterol binding is required for induction of host responses and a recent study has shown that a wheat LTP competes with elicitor for a low abundance, high affinity site on tobacco plasma membranes³¹. These results strengthen our hypothesis that *DIR1*/LTP may bind a lipid molecule and suggest that a plasma membrane receptor may also play a role in *DIR1*-mediated long distance signaling during SAR. □

Methods

Plant growth

Transfer DNA tagged lines of *Arabidopsis thaliana* ecotype Wassilewskija (Ws) were obtained from Ohio State University (K. Feldman Collection). Seeds were surface-sterilized and germinated on Murashige & Skoog (MS) medium for 7 to 10 days under continuous light, then transferred to soil (Sunshine Mix #1, Sun Gro Horticulture) hydrated with 1 g l^{-1} 20–20 fertilizer and grown for 3 weeks at 22°C under a 9-hour photoperiod ($150\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$).

Bacterial growth and inoculation

Pseudomonas syringae pv. *tomato* DC3000 strains (rifampicin resistance on the chromosome and kanamycin resistance on plasmids pVSP61 or pV288[*avrRpt2*]) were obtained from A. Bent. *Pseudomonas* pv. *maculicola* was obtained from F. Ausubel and contained the plasmid pLAFR3 $\pm$ *avrRpm1*. *Pst* or *Psm* from overnight log phase cultures were diluted to 10^5 or 10^6 CFU ml^{-1} in 10 mM MgCl_2 , pressure infiltrated into leaves and bacterial growth in plants determined as described²⁰. Plant inoculations were initiated about 25 days after seeds were placed on MS media.

SAR assay

SAR was monitored by comparing growth of virulent *Pst* in plants induced for SAR (AV treatment), to growth in uninoculated plants (MV treatment). Plants were either induced by inoculation in one or two leaves with avirulent *PstavrRpt2* (10^6 CFU ml^{-1}) or mock-inoculated with 10 mM MgCl_2 and two to three days later challenged by inoculation of other leaves with the isogenic virulent *Pst* strain (10^5 CFU ml^{-1}). A control MM treatment (mock-inoculation with 10 mM MgCl_2 on one leaf; 2 days later a second mock-inoculation on other leaves) was performed to test for contamination of the leaf surface or MgCl_2 solution. The Student's *t*-test was used to determine the significance of differences in bacterial growth between treatments.

RNA analysis

Total RNA was isolated from frozen leaf samples (3 to 4 leaves per sample) using the Total RNA Isolation Reagent (TRIzol, Gibco-BRL Life Technologies). RNA samples ($5\text{ }\mu\text{g}$) were separated by electrophoresis through formaldehyde-agarose gels and blotted to nylon membranes (Hybond-N, Amersham). *Arabidopsis* cDNA clones for *PR-1*, *PR-5* (provided by K. Lawton, Syngenta) and GST (*Arabidopsis* Biological Resource Center, Ohio State University, clone atts1553) were ^{32}P -labelled by random priming (Random Primer Kit, Amersham). Hybridizations and washes were carried out according to ref. 28. RNA loading and transfer was checked by hybridization with ubiquitin (*UBQ* in Fig. 4) and *rDNA* probes.

Petiole exudates

This method was modified from previously published methods^{29,30}. Ws and *dir1-1* plants were induced for SAR by inoculation with *PstavrRpt2* (10^6 CFU ml^{-1}). Ten to twelve hours later (start of the oxidative burst³), petioles were cut above the stem, surface sterilized for 10 s (50% ethanol, 0.0006% bleach), rinsed in sterile 1 mM EDTA and then submerged in $\sim 1.7\text{ ml}$ of 1 mM EDTA and $50\text{ }\mu\text{g ml}^{-1}$ ampicillin (Sigma). The sterilization step must be long enough to kill phylloplane bacteria, but not long enough to kill bacteria in the plant intercellular spaces. Exudates (5–10 leaves per microtube) were collected over 2 days. The exudates were diluted twofold then infiltrated into naïve healthy Ws or *dir1-1* plants. Infiltrated leaves (3 to 4) were collected one day later for *PR-1* gene expression analysis. Exudates were tested for sterility by plating $100\text{ }\mu\text{l}$ on King's B supplement with rifampicin and kanamycin⁷ and no contamination was detected. Exudate samples (1.7 ml) contained between 50 to $100\text{ }\mu\text{g}$ total protein (Biorad Protein Assay Kit).

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Competing interests statement

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A cryptic protease couples deubiquitination and degradation by the proteasome

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The 26S proteasome is responsible for most intracellular proteolysis in eukaryotes^{1,2}. Efficient substrate recognition relies on conjugation of substrates with multiple ubiquitin molecules and recognition of the polyubiquitin moiety by the 19S regulatory complex—a multisubunit assembly that is bound to either end of the cylindrical 20S proteasome core. Only unfolded proteins can

pass through narrow axial channels into the central proteolytic chamber of the 20S core, so the attached polyubiquitin chain must be released to allow full translocation of the substrate polypeptide. Whereas unfolding is rate-limiting for the degradation of some substrates and appears to involve chaperone-like activities associated with the proteasome^{3–5}, the importance and mechanism of degradation-associated deubiquitination has remained unclear. Here we report that the POH1 (also known as Rpn11 in yeast) subunit of the 19S complex is responsible for substrate deubiquitination during proteasomal degradation. The inability to remove ubiquitin can be rate-limiting for degradation *in vitro* and is lethal to yeast. Unlike all other known deubiquitinating enzymes (DUBs) that are cysteine proteases^{6,7}, POH1 appears to be a Zn²⁺-dependent protease.

To study the steps of proteasomal degradation that occur after unfolding, we developed a substrate containing an amino-terminal ubiquitin fused to the ovomucoid first domain⁸. We used this substrate because it is amenable to unfolding and fluorescent labelling. The ovomucoid moiety (OM) was unfolded irreversibly by disulphide bond reduction and alkylation of six cysteines with a fluorophore, lucifer yellow (Fig. 1a). The ubiquitin–ovomucoid (UbOM) substrate also included an N-terminal six-His tag and a haemagglutinin (HA) epitope at the ubiquitin–ovomucoid junction.

We mutated G76 to V76 in ubiquitin, as this mutation (G76V) can prevent release of ubiquitin by DUBs⁹. Ub(G76)OM is degraded rapidly by the 26S proteasome in an ATP-dependent manner (Fig. 1b). When we compared the degradation kinetics of UbOM with ovomucoid alone (Fig. 1c), we found that both are degraded with the same Michaelis constant (K_m), indicating that these substrates are targeted through the unfolded ovomucoid moiety and supporting previous conclusions that monoubiquitin does not bind well to proteasomes. However, on mutation of G76 to V76 in ubiquitin (Ub(V76)OM), the catalytic rate constant (k_{cat}) was reduced tenfold, whereas K_m was unchanged. Thus, the ubiquitin moiety appeared to have a role in a post-targeting step of proteasomal processing.

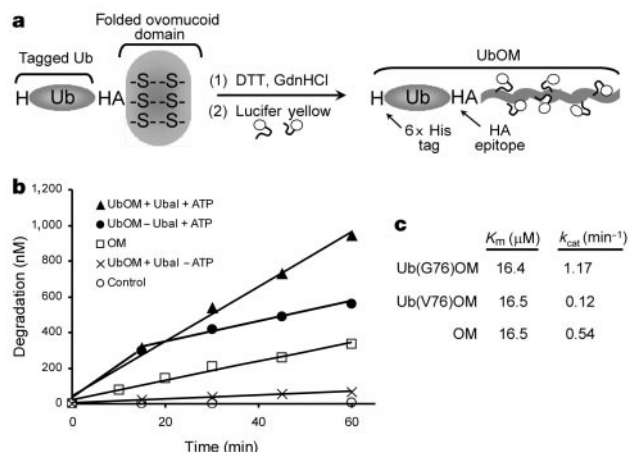


Figure 1 Proteasomal degradation of UbOM is targeted by means of the unfolded ovomucoid moiety. **a**, Scheme for preparation of unfolded, fluorescently labelled protein substrates. DTT, dithiothreitol; GdnHCl, guanidine HCl; Ub, ubiquitin. **b**, Degradation is ATP-dependent. Reactions contained 16 μ M ovomucoid (OM) or Ub(G76)OM, with or without (control) bovine 26S proteasomes and 2 μ M ubiquitin-aldehyde (Ubal). **c**, The G76V mutation at the ubiquitin–ovomucoid junction slows degradation by tenfold. The low k_{cat} of ovomucoid relative to Ub(G76)OM is because the fused ubiquitin blocks engagement of the ovomucoid N terminus by the proteasome; such N-terminal engagement reduces the rate and processivity of degradation (T.Y. and R.E.C., unpublished results).

The different degradation rates of the UbOM substrates suggested that Ub(G76)OM was deubiquitinated during degradation whereas with Ub(V76)OM the ubiquitin moiety was degraded together with the unfolded OM extension. Figure 2a shows that this was indeed the case. Degradation of Ub(G76)OM, but not Ub(V76)OM, generated a product that was recognized by anti-ubiquitin but not anti-HA antibody. We isolated this product on Ni²⁺ resin and determined by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry that its molecular mass was within 8 Da of six-His-tagged ubiquitin (data not shown). Thus, the ubiquitin moiety was processed precisely at G76. For two reasons, these results indicated the action of an unusual DUB. First, the degradation reactions contained 2 μ M ubiquitin-aldehyde (Ubal), a potent DUB inhibitor¹⁰. Pre-incubation of purified proteasomes with Ubal eliminated deubiquitination of K48-linked Ub₂ and ubiquitin Asp 77, whereas even fivefold more Ubal did not inhibit UbOM degradation (data not shown). Therefore, Ub(G76)OM is processed by a Ubal-insensitive DUB. Second, deubiquitination by this activity promotes degradation (Fig. 1c). When incubated without Ubal, Ub(G76)OM was rapidly deubiquitinated (probably by UCH37 (ref. 11)) and then degraded slowly (that is, at the same rate as ovomucoid alone; Fig. 1b).

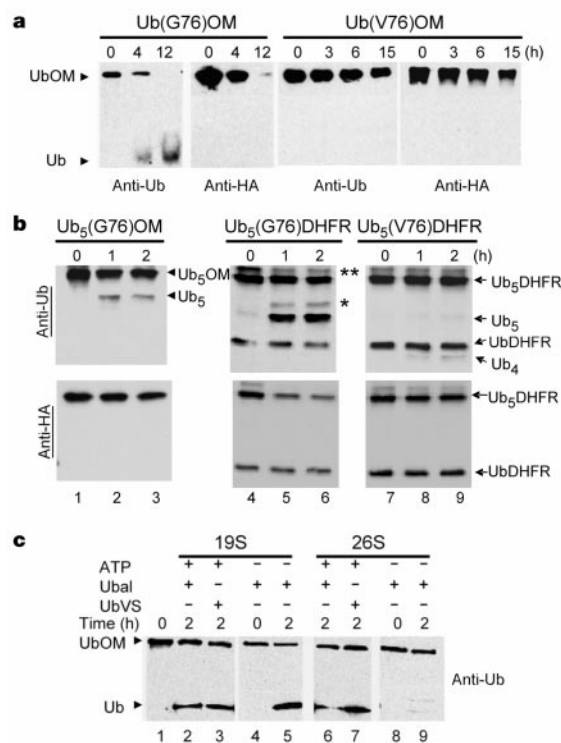


Figure 2 A ubiquitin-aldehyde (Ubal)-insensitive DUB couples release of ubiquitin with substrate degradation (western blots). **a**, Ubiquitin was generated on degradation of Ub(G76)OM but not Ub(V76)OM. A total of 0.5 μ M Ub(G76)OM or 16 μ M Ub(V76)OM was incubated with bovine 26S proteasomes (42 nM or 420 nM, respectively) and Ubal. **b**, Polyubiquitin release accompanies degradation of either folded (DHFR) or unfolded (OM) conjugates. Reactions included bovine proteasomes, Ubal, and 3 μ M of the indicated substrate. Ub₅ was produced on degradation of the Ub₅(G76) conjugates, whereas Ub₄ was generated from Ub₅(V76)DHFR. The DHFR substrates also contained about 10% UbDHFR and a small amount of Ub₆DHFR (double asterisk). Ub₆ release (asterisk) accompanied Ub₅(G76)DHFR degradation, but Ub₅(V76)DHFR degradation was negligible and yielded no detectable Ub₅ or Ub₆. As neither Ub(V76)DHFR nor Ub(G76)DHFR is targeted to the proteasome, their levels remained constant. **c**, The cryptic DUB is ATP-dependent in bovine 26S proteasomes, but ATP-independent in the isolated 19S complex. Assays used 2 h incubation with 3 μ M Ub(G76)OM. UbVS, ubiquitin vinylsulphone.

Because this deubiquitinating activity was observed only with a proteasomal degradation substrate, we named it the 'cryptic DUB'.

The properties of the cryptic DUB suggested that it could be responsible for polyubiquitin release from conjugates during degradation. To investigate this possibility, we prepared protein conjugates containing a pentaubiquitin chain (Ub₅) by conjugation of K48-linked tetraubiquitin (Ub₄) to K48 of the ubiquitin in the fusion proteins³. As shown in Fig. 2b (lanes 1–3), Ub₅ was released from Ub₅(G76)OM incubated with 26S proteasomes in the presence of Ubal. We confirmed that the ovomucoid moiety was degraded by measuring the generation of trichloroacetic acid (TCA)-soluble fluorescence. Similar results were observed with a folded substrate, Ub₅(G76)DHFR (Fig. 2b, lanes 4–6). A related substrate, Ub₅(V76)DHFR, is targeted efficiently to the proteasome ($K_m = 35$ nM), but its degradation is slow ($k_{cat} = 0.05$ min⁻¹) and is accompanied by degradation of the proximal mutant ubiquitin Ub(V76)³. These properties are reminiscent of our findings with Ub(V76)OM. Indeed, comparison of Ub₅(G76)DHFR and Ub₅(V76)DHFR, in which DHFR contained a carboxy-terminal HA-tag⁹, showed that the former substrate was degraded more rapidly (Fig. 2b, lanes 4–6, lower panel) and with release of Ub₅ (lanes 4–6, upper panel). In contrast, degradation of Ub₅(V76)DHFR was slow (lanes 7–9) and Ub₄ rather than Ub₅ was produced, consistent with degradation of Ub(V76). With both substrates, the DHFR moiety was degraded (that is, free HA-tagged DHFR was not detected). Thermodynamically, ubiquitin is exceedingly stable¹², and the need to unfold Ub(V76) before its degradation may contribute to the slow turnover of the Ub(V76)-containing substrates.

Deubiquitination of UbOM by the cryptic DUB in 26S proteasomes is ATP-dependent (Fig. 2c, lanes 1, 6, 8 and 9), and ATPγS could not substitute for ATP (not shown). However, Ubal-insensitive deubiquitination by the isolated 19S complex is ATP-independent (lanes 1, 2, 4, 5). Similar results were obtained with the irreversible DUB inhibitor ubiquitin vinylsulphone (UbVS)¹³ (lanes 3, 7) or the Ub₅(G76)OM substrate (data not shown). Thus, ATP hydrolysis is not required for deubiquitination *per se*; rather, the ATP-dependence of the cryptic DUB must originate from other substrate processing step(s). The timing of deubiquitination during substrate processing is critical: the polyubiquitin chain needs to fulfil its targeting function, but not impede subsequent substrate translocation. The ATP-dependence seen with intact proteasomes probably reflects coupling of deubiquitination to degradation, which may insure proper timing of polyubiquitin release by controlling the position of the proximal ubiquitin at the DUB active site. To date, ATP hydrolysis has been found to have a role in productive polyubiquitin chain binding¹⁴, substrate unfolding⁴, and opening of the channels that lead into the 20S proteasome¹⁵; polypeptide translocation may also require ATP. It is unclear at

present to which step deubiquitination is coupled.

Unlike other DUBs, the activity of the cryptic DUB is insensitive to Ubal or UbVS. The fact that similar rates of deubiquitination were observed with the 19S complex and the intact 26S proteasome (Fig. 2c) suggests that a subunit of the 19S complex is responsible. Three proteasome-associated DUBs are known. UCH37 is a subunit of the 19S complex that disassembles ubiquitin conjugates¹¹. However, unlike the cryptic DUB, its activity is restricted to the distal rather than proximal ubiquitin of a polyubiquitin chain, and it is completely inhibited by Ubal. Ubp6/USP14 is a DUB of unknown specificity that can associate with the proteasome^{13,16}, but Ubp6 is largely inhibited by Ubal or UbVS (ref. 13, and our own unpublished data). Moreover, although the bovine 19S and 26S complexes contained about 20-fold different levels of Ubp6 (0.005 and 0.12 molecules per complex, respectively; data not shown), they displayed similar Ubal-insensitive DUB activity (Fig. 2c). Ubp6 was excluded definitively as the cryptic DUB by comparison of 26S proteasomes purified from wild-type and *ubp6Δ* yeast; both contained the Ubal-insensitive and ATP-dependent deubiquitination activity (Supplementary Information Fig. S1). Doa4 is a yeast DUB originally thought to participate in substrate processing by the proteasome because its deficiency leads to accumulation of small peptides linked to mono- or polyubiquitin¹⁷. However, the ability of Doa4 to hydrolyse linkages between ubiquitin molecules¹⁷ and its substoichiometric association with the 26S proteasome¹⁸ make it unlikely that Doa4 is the cryptic DUB.

What other protein might account for the cryptic DUB activity? The 19S regulatory complex is composed of two subcomplexes: the base, which includes Rpn1, Rpn2 and six ATPases, and the lid, which contains at least eight non-ATPase subunits¹⁹. Among the non-ATPases, Rpn11 (POH1 in humans) is the most conserved subunit, with 65% identity between the yeast and the human proteins (Fig. 3). This high conservation suggested that a catalytic activity might be associated with Rpn11. Eukaryotes contain another multiprotein complex, the COP9 signalosome (CSN), whose eight subunits are markedly similar to those of the proteasome lid¹⁹. The CSN recently was shown to contain a deconjugation activity specific for the ubiquitin-like protein Nedd8 (ref. 20). Thus, we hypothesized that Rpn11/POH1 is the cryptic DUB, and that yeast Rri1 (JAB1 in humans), the CSN orthologue, is responsible for de-conjugation of Nedd8. Proteasome subunits Rpn11/POH1 and Rpn8/S12, CSN subunits Rri1/JAB1, and also eIF3 subunits p40 and p47 each contain an MPN domain²¹. We identified nine amino acids within this domain (Fig. 3) as potential catalytic site residues of a protease; importantly, eight of these are conserved between Rpn11 and JAB1, but not between Rpn11 and S12 or eIF3 p40.

We predicted that loss of activity of the cryptic DUB would severely impede proteasomal degradation and be lethal to the cell. Therefore, we tested the abilities of *rpn11* mutants to support



Figure 3 Alignment of the MPN domain of MPN family proteins. Nine positions within Rpn11 (denoted by an asterisk or a triangle) were subjected to mutagenesis in a search for active site residues; a triangle indicates where conservative substitutions (that is, H109Q,

H111Q and D122E) were found to be lethal in yeast. Residues that are identical or similar to the consensus sequence are shaded black or grey, respectively (CLUSTAL W).

growth of yeast (Fig. 4a). Mutant alleles of *rpn11* tagged with protein A at the C terminus (*rpn11-ProA*) were cloned into a low-copy plasmid under the control of the native *RPN11* promoter. These plasmids were transformed into yeast that had the chromosomal copy of *RPN11* deleted but carried a wild-type *RPN11* (tagged with HA at the C terminus) on a low-copy *URA3* plasmid. When cured of the *URA3* plasmid by transfer to plates containing 5-fluoro-orotic acid (5-FOA), these yeast cells were forced to rely on *rpn11-ProA* for growth. Figure 4a shows that among nine mutants, five of them support growth as well as the wild type. However, the H111Q, H109Q and D122E mutations are lethal, and D142A only partially supports growth. Overexpression of the latter four mutant alleles together with wild-type *RPN11* leads to slowed growth and reduced cell density in stationary phase (Supplementary Information Fig. S2). Moreover, we observed elevated levels of ubiquitin conjugates in these strains (Fig. 4b), an indication of defective proteasomes. To exclude the possibility that the lethality of the mutations was due to impaired proteasome assembly, we isolated 26S proteasomes from yeast strains that harboured low-copy plasmids to express wild-type Rpn11-HA together with each of the mutant Rpn11-ProA proteins. Immunoblotting with anti-protein A and anti-HA antibodies revealed that both wild-type and mutant Rpn11 proteins were present (Fig. 4c). Note that wild-type Rpn11-protein A was

incorporated at a higher level than the mutated forms. This correlated with higher levels of the wild-type fusion protein in the cell lysates (data not shown). It is possible that the uncomplexed mutant Rpn11 subunits were intrinsically unstable, or that dysfunctional proteasomes containing inactive Rpn11 were degraded selectively.

The above results suggest that Rpn11 is the cryptic DUB and that H109, H111 and D122 are active-site residues; D142 may also assist catalysis. Importantly, the viability of the C116S mutant excludes a cysteine protease mechanism; this is despite similarity of residues 113–121 in Rpn11 with the catalytic Cys-box of the ubiquitin-specific protease (UBP) family of DUBs²². Rather, the identities of the critical residues suggest that Rpn11 is a Zn^{2+} -dependent metalloprotease²³. When bovine 19S complex was incubated with the Zn^{2+} chelator TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine), deubiquitination of Ub(G76)OM was inhibited; this effect was prevented when TPEN was pre-incubated with excess Zn^{2+} (Fig. 4d). TPEN did not inhibit the cryptic DUB in intact bovine 26S proteasomes (data not shown), possibly because the 19S and 26S complexes differ with respect to stability of the lid subcomplex or access to the Rpn11 active site. An ATP-dependent, Ubal-insensitive DUB activity in 26S proteasomes that is inhibited by o-phenanthroline has been reported previously²⁴. We think this activity probably corresponds to the cryptic DUB identified here. However, we found that inhibition by o-phenanthroline, which is not prevented by excess Zn^{2+} , is apparently due to a general structural disruption of both the 19S and 26S complexes (data not shown).

Thus far, our attempts to generate an active deubiquitinating enzyme from Rpn11 expressed in *Escherichia coli* or *in vitro* have been unsuccessful. Rpn11 may be unable to fold as an isolated subunit into a native, active conformation. However, substrate interactions with neighbouring subunits in the 19S complex are probably required to position conjugates for cleavage by Rpn11. Normally, these interactions would involve a portion of the substrate protein that is unfolded and near the (poly)ubiquitin-protein attachment site. In the case of the Ub₅(V76)DHFR substrate, the proximal ubiquitin was itself unfolded and translocated until the next ubiquitin, which possessed a G76 residue, was in position for cleavage and Ub₄ was released.

Except for residues 113–121 (see above), Rpn11/POH1 bears little resemblance to either ubiquitin C-terminal hydrolase (UCH)- or UBP-type DUB enzymes²⁵. Rpn11/POH1 is also unique among known DUBs in that it is essential for growth, and its discovery helps to place deubiquitination both functionally and mechanistically in the scheme of proteasomal degradation. MPN domain proteins are represented in all three biological domains of life (Archaea, Bacteria and Eukaryota)^{21,26}. The properties of Rpn11/POH1 provide insight into the possible functions and mechanisms of those MPN proteins that contain the putative Zn^{2+} -binding site. In particular, our results suggest that JAB1 in the COP9 signalosome is responsible for de-conjugation of Nedd8. Another Rpn11 homologue of interest is C6.1A, the aberrant expression of which has been implicated in cases of T-cell leukaemia²⁷. □

Methods

Strains and plasmids

The UbOM plasmid was constructed by putting a six-His-tagged ubiquitin²⁸ between *NdeI* and *HindIII* sites of vector pRSet, then inserting HAOM (made by polymerase chain reaction (PCR) from pOMCH11-X (ref. 8) and a primer encoding an HA epitope) at the *HindIII* site. The Ub(G76)DHFR plasmid was derived from Ub(V76)DHFR³ by site-directed mutagenesis. Wild-type *RPN11* was cloned into pRS316 with 200 base pairs of upstream sequence and the addition of a C-terminal HA tag. *RPN11-ProA* was cloned from the yeast sDL73 (ref. 16) and put into pRS313 with a 200-bp upstream sequence of *RPN11*, or into pESC-LEU (Stratagene) without the upstream sequence. Mutations were introduced into those plasmids by site-directed mutagenesis. A *RPN11/rpn11Δ* diploid strain (Research Genetics, strain number 25683) was transformed with pRS316 *RPN11-HA*, sporulated, and tetrads dissected to obtain haploid strain RCY519 that was *rpn11Δ*[pRS316-*RPN11-HA*].

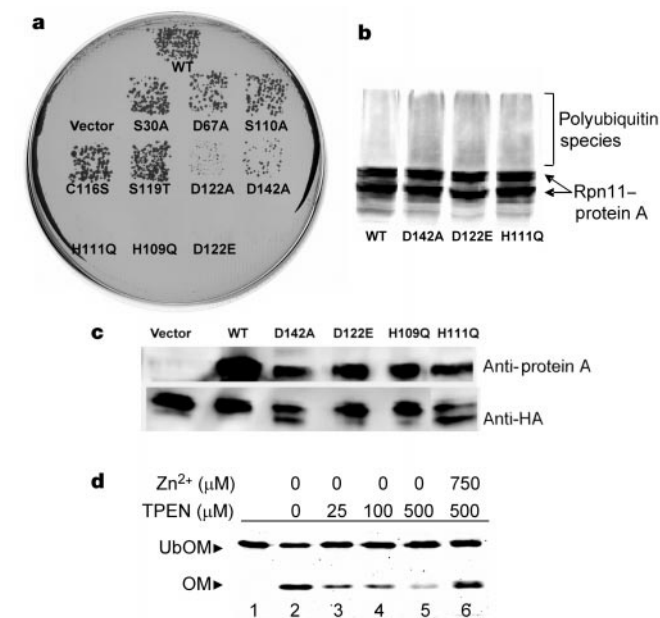


Figure 4 Evidence that Rpn11/POH1 is a Zn^{2+} -dependent DUB and characterization of *rpn11* mutants. **a**, Conservative changes in Rpn11 at H109, H111 and D122 were lethal and identify potential Zn^{2+} -binding active-site residues. Growth on 5-FOA plates was assessed after 4 days at 30 °C for *rpn11Δ*[pRS316-*RPN11-HA*] yeast that contained single-copy (pRS313) plasmids with the indicated *rpn11-ProA* alleles. WT, wild type. **b**, Overexpression of *rpn11* mutants leads to accumulation of ubiquitin conjugates. Lysates from stationary phase yeast (Supplementary Information Fig. S2) were analysed by anti-ubiquitin western blot. Loading controls are provided by the full-length and partially proteolysed Rpn11-ProA fusions, which bind the antibodies by their protein A extensions. **c**, Mutant Rpn11 subunits are incorporated into proteasomes. Wild-type Rpn11-HA was expressed in each strain (see **a**) together with the indicated wild-type or mutant Rpn11-ProA fusion (or empty vector). Gel-purified proteasomes from these yeast cells were analysed by western blot. The doublet seen with some Rpn11-HA species is an artefact from loading the gel slices. **d**, Inhibition of the Ubal-insensitive DUB by a Zn^{2+} chelator. Bovine 19S complex was pre-incubated for 10 min at 37 °C with increasing amounts of TPEN or with TPEN plus excess Zn^{2+} . After incubation with 3 μ M Ub(G76)OM and SDS-PAGE, the remaining substrate and the released ovomucoid proteins were detected by their fluorescence.

Proteasome isolation

26S proteasomes were purified from bovine erythrocytes²⁹, and the bovine 19S complex (PA700) was a gift from G. DeMartino. Yeast 26S proteasomes were affinity-purified using sDL73 as the wild type¹⁶ or a *ubp6Δ* derivative of sDL73 made by standard techniques. For *rpn11* mutant proteasomes, strain RCY519 was transformed with various pRS313 *rpn11-ProA* mutants and grown to stationary phase in SC (–URA, –LEU) medium. Lysates were prepared in 50 mM HEPES, pH 8, containing 5 mM MgCl₂, 2 mM ATP, 1 mM EDTA, 0.3 M sorbitol, 0.1 M NaCl, 40 μM chymostatin, and 1 × protease inhibitor cocktail (Sigma, number P8215). After 1 h centrifugation at 100,000g, the supernatant was further centrifuged at 400,000g for 1 h and the clear pellets were dissolved in degradation assay buffer (see below); after separation by non-denaturing polyacrylamide gel electrophoresis (PAGE), proteasomes were detected by soaking the gel in Suc-LLVY-AMC¹⁹. Gel pieces containing the 26S species were excised, heated with SDS sample buffer, and analysed by SDS–PAGE and immunoblotting.

Substrate proteins

Recombinant UbOM (G76 or V76) and UbDHF (G76 or V76) were expressed in *E. coli* and purified with Ni²⁺-NTA agarose (Qiagen) followed by gel filtration (Superdex 75). Ovomucoid and UbOM were unfolded by 6 M guanidine-HCl and 1 mM dithiothreitol (DTT) in 50 mM Tris-HCl pH 7.4, 0.5 mM EDTA, then exchanged into buffer containing 1 mM tris(2-carboxyethyl)phosphine-HCl instead of DTT. Lucifer yellow iodoacetamide (Molecular Probes) was added in ninefold excess over cysteines. After 5 h in the dark at 20 °C, free dye was removed by desalting (Sephadex G25); ≥80% of the cysteines were modified. Ub₂ chains prepared from UbC48 and UbD77 (ref. 30) were transferred to monoubiquitin substrates³ and the Ub₂ protein products were purified on Ni²⁺-NTA agarose.

Degradation assays and immunoblots

Conditions for *in vitro* degradation or deubiquitination were essentially as described³. Unless specified otherwise, reactions were at 37 °C and included 42 nM 26S proteasomes or 19S complex and 2 μM Ubal. Proteasomes were pre-incubated with Ubal for 5 min before addition of substrates; 10 U ml^{–1} apyrase (Sigma) was used for ATP depletion. To quantify degradation of lucifer yellow-labelled substrates, samples were precipitated with 20% TCA, centrifuged, and fluorescence in the supernatant was measured (426 nm excitation, 531 nm emission) after neutralization with Tris base. Immunoblotting followed standard protocols and detection was by means of chemiluminescence (Pierce). The antibodies used were: rabbit polyclonal affinity-purified anti-ubiquitin, anti-protein A (Sigma), and monoclonal anti-HA (clone 16B12; Covance).

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Active genes are tri-methylated at K4 of histone H3

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Lysine methylation of histones *in vivo* occurs in three states: mono-, di- and tri-methyl¹. Histone H3 has been found to be di-methylated at lysine 4 (K4) in active euchromatic regions but not in silent heterochromatic sites². Here we show that the *Saccharomyces cerevisiae* Set1 protein can catalyse di- and tri-methylation of K4 and stimulate the activity of many genes. Using antibodies that discriminate between the di- and tri-methylated state of K4 we show that di-methylation occurs at both inactive and active euchromatic genes, whereas tri-methylation is present exclusively at active genes. It is therefore the presence of a tri-methylated K4 that defines an active state of gene expression. These findings establish the concept of methyl status as a

determinant for gene activity and thus extend considerably the complexity of histone modifications.

The amino-terminal tails of histones are subject to a variety of modifications. The precise pattern of histone modifications in the chromatin surrounding a gene are thought to control transcriptional activity^{3,4}. Methylation of lysines is one such modification that occurs on histones H3, H4 and H2B.

Proteins with SET domains have the potential to methylate lysines in the N-terminal tail of histones. One example is the SUV39H1 protein, which methylates specifically lysine 9 of H3 (K9 H3) and represses transcription through the recruitment of HP1 (refs 5–7). In an effort to identify new methylases, we decided to characterize SET proteins from *S. cerevisiae*, as this yeast has no detectable H3 K9 methylation. Five of the six SET domain proteins in yeast (Set1, Set2, Set3, Set4, Set6) were tagged, purified and analysed for methylase activity. Set1 and Set2 were found to methylate histone H3, whereas the other SET proteins were inactive (see Supplementary Fig. 1). Figure 1a shows that purified Set1 is able to methylate histone H3 at K4. Purification of a Set1 protein that lacks part of the SET domain, Set1ΔC, is unable to methylate H3 (Fig. 1b), even though it associates with all the proteins shown to be part of the Set1 complex (refs 8, 9 and data not shown). These results point to Set1 as the enzyme responsible for the K4 H3 methylation within the Set1 complex.

The ε-amino group of lysine residues may be mono-, di- or tri-methylated. We considered the possibility that the different states of methylation may provide a further level of regulation for transcription. To probe this hypothesis we raised antibodies that specifically recognize either the di-methylated or tri-methylated state of K4 H3. Figure 2a left panel shows that an antibody raised against di-methylated K4 recognizes H3 from a purified source of histones and is effectively competed by a di-methylated but not a tri-methylated K4 H3 peptide. An antibody raised against tri-methylated K4 is specifically competed by the tri- but not di-methylated H3 peptide (right panel). Three independent lines of evidence further

testify to the specificity of the di- and tri-methylated K4 H3 antibodies. First, they selectively recognize histone H3 in a total yeast extract (wild type, Fig. 2b). Second, they do not recognize H3 from a yeast strain containing a K4 H3 mutation to alanine (K4A, Fig. 2b). Last, they do not recognize bacterially produced recombinant, un-methylated H3 (Fig. 2c).

We next asked whether the Set1 enzyme is able to catalyse both the di- and tri-methylated state of K4. Figure 2c shows that both di-methylated and tri-methylated K4 antibodies can recognize recombinant histone H3 after it has been methylated by Set1. Indeed, Fig. 2d shows that Set1 is the only SET protein that can carry out these reactions in yeast. Deletion of the *SET1* gene

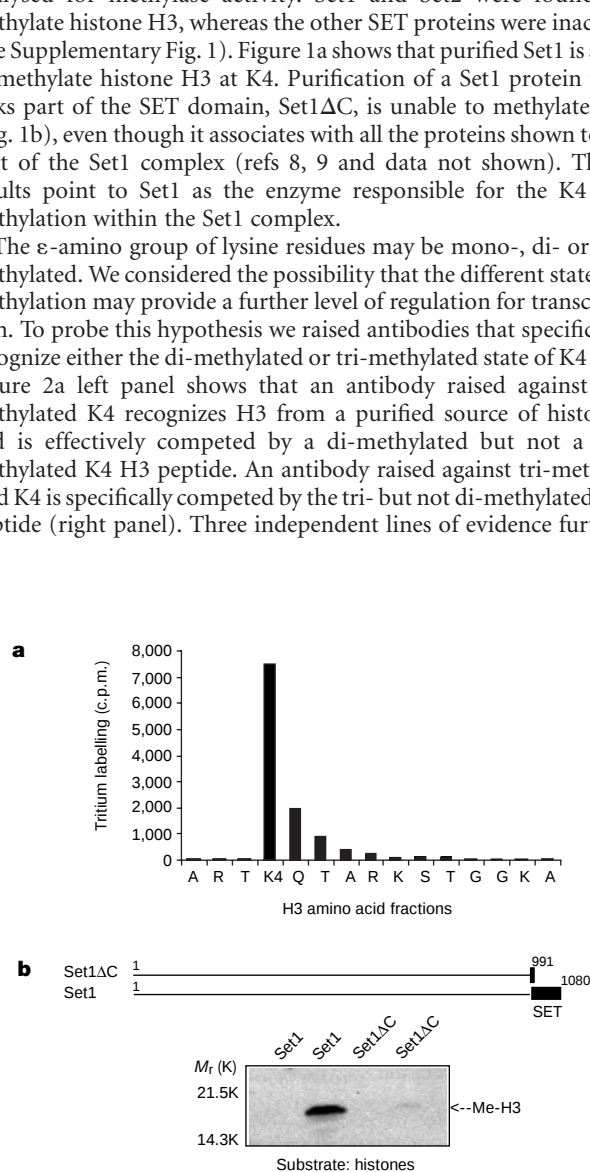


Figure 1 Set1 methylates histone H3. **a**, Mapping the Set1 methylation site in H3. Recombinant H3 was radioactively methylated by PTA-Set1 and sequenced by sequential Edman degradation. Fractions corresponding to each amino acid were collected and monitored by liquid scintillation counting. The sequence of H3 is indicated below the fractions. **b**, The SET domain of Set1 is responsible for its methylase activity. Identical amounts of wild-type Set1 and Set1ΔC purified from yeast were assayed for methylase activity (see Methods). The reaction products were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and viewed after autoradiography.

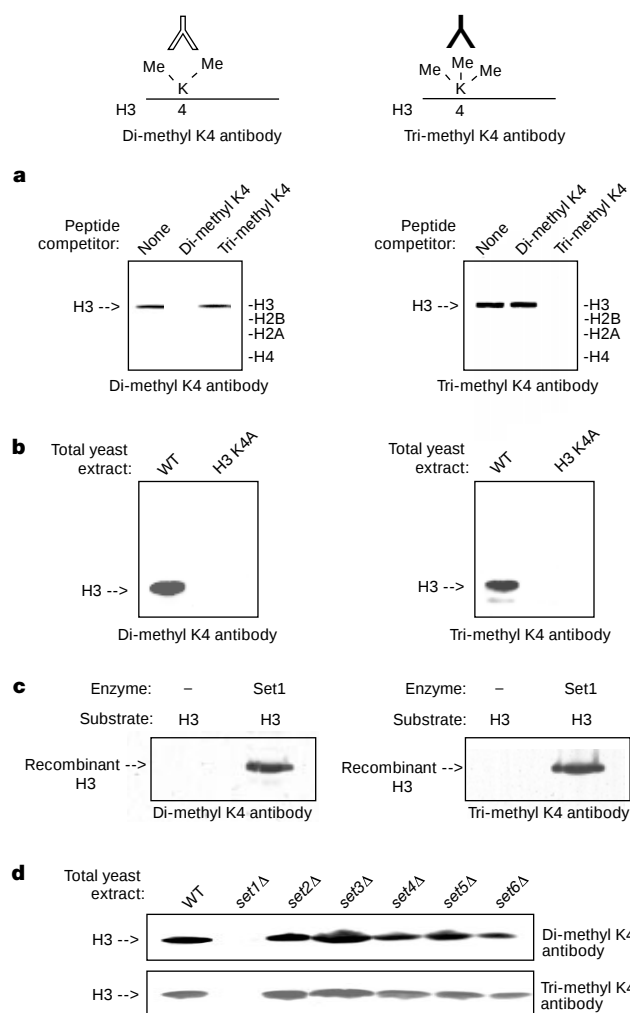


Figure 2 Specific antibodies discriminate between H3 K4 di-methyl and tri-methyl. Inset at the top shows the structure of the di- and tri-methylated K4 antibodies. **a**, Total histones from calf thymus (Sigma) were resolved by SDS–PAGE, blotted to nitrocellulose and probed with either a di- or a tri-methylated K4 antibody as indicated. Peptide competition where indicated was at 0.1 μg ml^{−1}. **b**, The di-methylated K4 and tri-methylated K4 antibodies specifically recognize H3 K4 methylation in a total yeast extract. Equivalent amounts of total extract from wild type and H3 (K4A) mutant strains were western blotted and probed with the di- or tri-methylated K4 antibodies, as indicated. **c**, Set1 is responsible for di-methylation and tri-methylation of H3 K4 *in vitro*. Recombinant unmethylated H3 was methylated by Set1 and the reaction products were western blotted with the di-methylated K4 and tri-methylated K4 antibodies. **d**, Set1 is responsible for di- and tri-methylation of H3 K4 *in vivo*. Identical amounts of total extract from wild type and SET knockout isogenic strains were western blotted and probed with the di-methylated and tri-methylated K4 antibodies.

abolishes recognition of histone H3 by the di- and tri-methylated K4 antibodies, whereas deletion of other *SET* genes (*SET2*, *SET3*, *SET4*, *SET5*, *SET6*) has no effect.

Evidence so far has identified Set1 as a repressor of transcription^{9–13}, yet the detection of di-methylated K4 H3 at potentially active euchromatic loci suggests that it may be linked to activation². Expression profiling of yeast deficient in Set1 was therefore carried out to establish whether it can activate transcription. This analysis identified 480 genes whose activity is significantly reduced in the absence of *SET1*. The activity of the top 20 genes (shown as Supplementary Fig. 3) is reduced by between 53% and 38% in the *set1Δ* strain relative to wild-type yeast. This indicates that Set1 is required for maximum expression levels of these genes but is not the only determinant in activity. Northern blot analysis of one of the genes regulated by Set1, *PPH3*, confirms that the messenger RNA expression is indeed reduced in the *set1Δ* strain (Fig. 3a). These results establish that Set1 has the ability to positively regulate transcription.

The genes identified by microarray analysis, such as *PPH3*, are constitutively active, as the expression profiling was done from yeast grown in rich medium. We also attempted to identify Set1 target genes whose activity can be regulated by signalling pathways. Figure 3b, c shows that the methionine-regulated gene *MET16* and the inositol-regulated gene *INO1* are positively regulated by Set1. Under conditions where these genes are expressed (without methionine or without inositol; Fig. 3b, c, respectively), deletion of *SET1* (lane 3) or mutation of histone H3 K4 to alanine (lane 4) reduces their

expression. The more severe effect of the K4A mutation on transcription may reflect the need for this residue for the binding of additional transcription regulators.

We next used the di-methylated and tri-methylated specific antibodies in chromatin immunoprecipitation (ChIP) analysis to establish whether the Set1-activated genes, identified in Fig. 3 (*PPH3*, *MET16* and *INO1*), were methylated at K4 H3. We used primers to analyse chromatin associated with the promoter (r1) or the coding region (r2).

Analysis of the *PPH3* gene (Fig. 4a) or another two genes identified in the *set1Δ* profiling (see Supplementary part 4) indicates that these constitutively active genes have both di-methylated and tri-methylated K4 H3 associated with their chromatin. For the *PPH3* gene the methylation status is the same in the promoter and coding region (r1 and r2). Thus, genes activated by the Set1 methylase have both di-methylation and tri-methylation of K4 H3.

Analysis of the methionine-regulated *MET16* gene shows that tri-methylated K4 only appears on the chromatin when the gene is active. In the repressed state, di-methyl is present and tri-methyl is absent (r1 and r2). When the gene is active, di-methyl remains (r2) or it is reduced (r1) whereas tri-methyl becomes apparent (r1 and r2).

The situation at the inositol-regulated *INO1* gene is much the same as at *MET16*. Under repressed conditions, di-methyl is present but tri-methyl is absent (r1 and r2). Under active conditions tri-methyl appears whereas di-methyl stays unchanged (r1 and r2). Two other inositol-regulated genes *INO2* and *INO4* also show the same features, with tri-methylated K4 H3 only appearing when the genes are active (Table 1 and data not shown).

The results of all the ChIP analyses are summarized in Table 1. Collectively, these results indicate that only the tri-methyl state of K4 H3 is unambiguously linked to Set1-dependent transcription. When genes are repressed, di-methylated K4 H3 is present but tri-methyl is absent. When genes are active, tri-methyl invariably appears and di-methyl often persists. The apparently ubiquitous presence of di-methylated K4 is consistent with previously reported findings at the chicken β -globin locus, where di-methylated K4 H3 is detected on large euchromatic regions encompassing both active and inactive genes¹⁴.

The role of di-methylated K4 H3 may be to determine a transcriptionally 'permissive' chromatin environment whereas the tri-methylated state may allow for an 'active' chromatin conformation. Whether a similar change in methyl status at K4 is operational in the instances where Set1 acts as a repressor is still to be determined. Given that Set1 is the only K4 methylating enzyme in yeast, it is worthwhile noting that di-methylation can take place in the absence of tri-methylation. This suggests that a mechanism exists that prohibits Set1 from adding a third methyl group to di-methylated K4. This may involve, for example, methyl-binding proteins protecting the di-methylated state, a distinct modification preventing the transition of di- to tri-methylated K4 or the action of a demethylase acting on the tri-methylated state.

Methylation at K4 (both di-methyl and tri-methyl) can be detected in both the promoter and the coding region of genes.

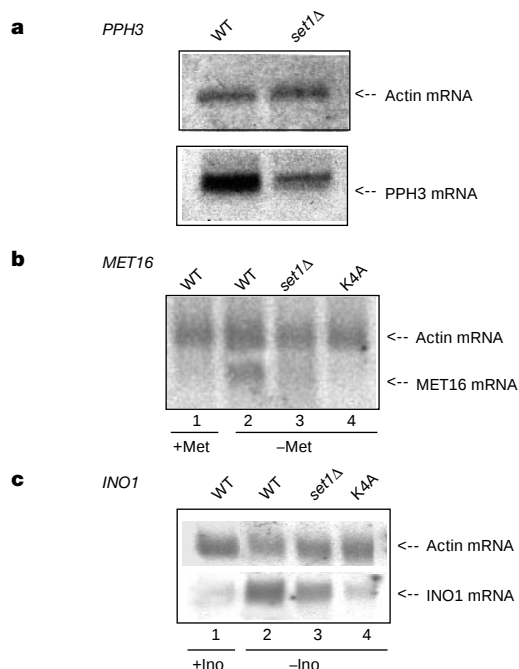


Figure 3 Set1 methylation is required for full gene expression. **a**, The *PPH3* mRNA level is downregulated in *set1Δ*. Northern analysis of *PPH3* and actin mRNA in a wild-type (UCC1001) and isogenic *set1Δ* strain. **b**, The *MET16* mRNA level is downregulated in *set1Δ* and the H3 (K4A) mutant. Northern blot analysis of *MET16* and actin mRNA was carried out in a wild-type (UCC1001) strain grown in the presence (30 mg l⁻¹) or absence of methionine (lanes 1 and 2, respectively). Northern analysis of *MET16* and actin mRNA was carried out in isogenic *set1Δ* and a H3 (K4A) mutant grown in the absence of methionine (lanes 3 and 4). **c**, The *INO1* mRNA level is downregulated in *set1Δ* and the H3 (K4A) mutant. Northern analysis of *INO1* and actin mRNA was carried out in a wild-type (UCC1001) strain grown in the presence (100 mg l⁻¹) or absence of inositol (lanes 1 and 2, respectively). Northern analysis of *INO1* and actin mRNA was carried out in isogenic *set1Δ* and a H3 (K4A) mutant grown in the absence of methionine (lanes 3 and 4).

Table 1 Summary of the K4 H3 methylation state of genes

Type of gene	Gene	Repressed	Active
Constitutively active genes	<i>PPH3</i>	—	Di and Tri
	<i>HAM1</i>	—	Di and Tri
	<i>NUP170</i>	—	Di and Tri
Inositol-regulated genes	<i>INO1</i>	Di	Di and Tri
	<i>INO2</i>	Di	Di and Tri
	<i>INO4</i>	Di	Di and Tri
	<i>MET16</i>	Di	Di and Tri

The growth conditions for activation and repression of the genes are described in the Methods section. Di, di-methylation detected in the gene; Tri, tri-methylation detected in the gene.

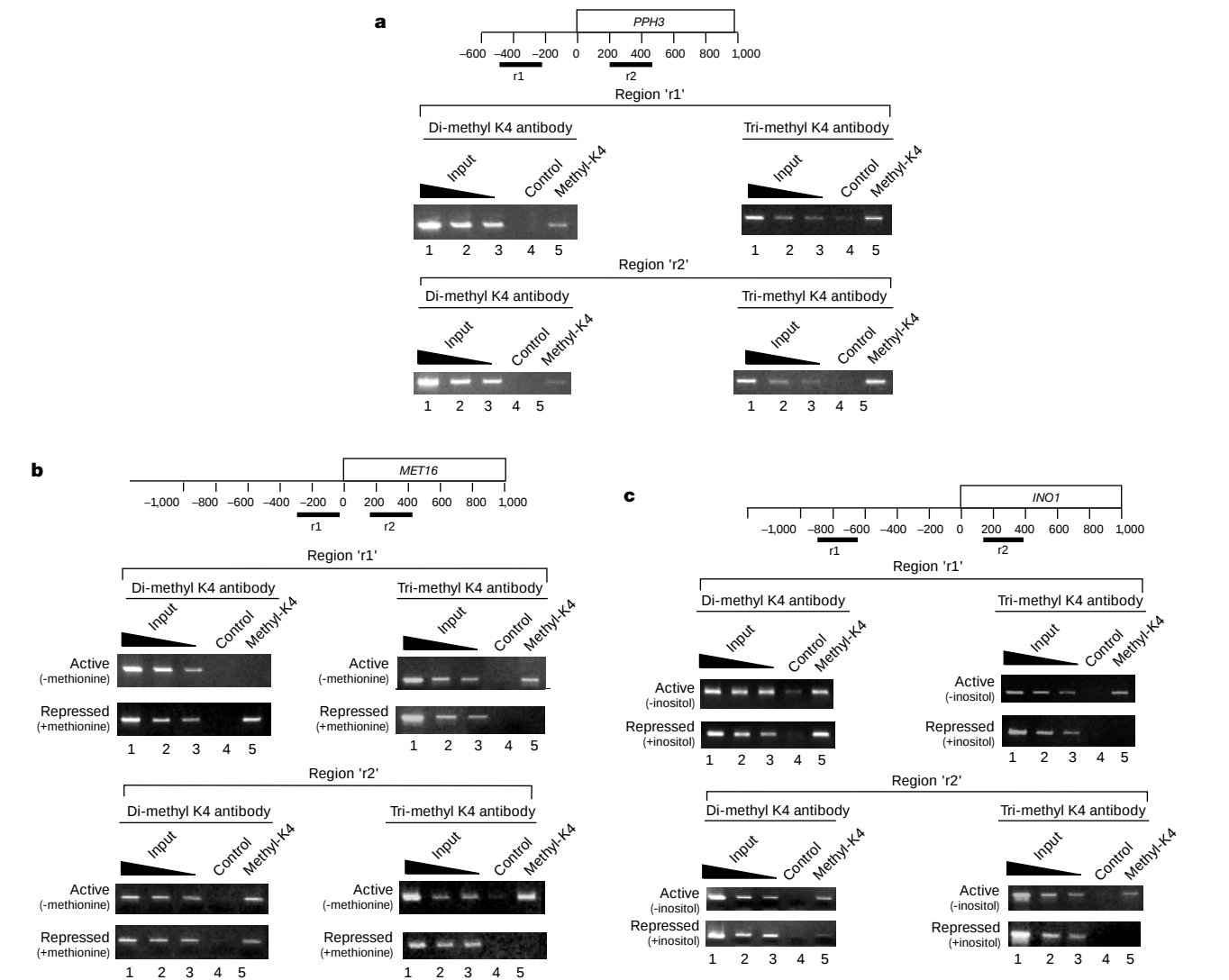


Figure 4 Di-methylation and tri-methylation of H3 K4 correlates with transcriptionally active genes. **a**, Chromatin from wild-type UCC1001 yeast grown in minimal medium (+inositol) was immunoprecipitated with di-methylated K4 and tri-methylated K4 antibodies and associated DNA was analysed by PCR using primers from within the *PPH3* promoter 'r1' and the coding region 'r2'. In each experiment, lanes 1, 2 and 3 are the PCR reactions performed on undiluted, 1:10 and 1:20 diluted input chromatin. Lane 4 is the PCR reaction from the chromatin immunoprecipitation performed with no antibody (negative control), whereas lane 5 is the PCR reaction when the specific antibody (as indicated) is used. **b**, Chromatin from UCC1001 wild-type yeast grown in minimal medium lacking methionine (–methionine) or supplemented with 30 mg l^{–1} methionine (+methionine) was immunoprecipitated with di- and tri-methylated K4 antibodies and

associated DNA was analysed by PCR using primers from within the *MET16* promoter r1 and the coding region r2. **c**, Chromatin from UCC1001 wild-type yeast grown in minimal medium lacking inositol (–inositol) or supplemented with 100 mg l^{–1} inositol (+inositol) was immunoprecipitated with di- and tri-methylated K4 antibodies and associated DNA was analysed by PCR using primers from within the *INO1* promoter r1 and the coding region r2. In each experiment, lanes 1, 2 and 3 are the PCR reactions performed on undiluted, 1:10 and 1:20 diluted input chromatin. Lane 4 is the PCR reaction from the chromatin immunoprecipitation performed with no antibody (negative control), whereas lane 5 is the PCR reaction when the specific antibody as indicated is used. In cases where no methylation was detected, the number of PCR cycles was increased from 30 up to 42 to confirm the lack of signal.

This finding is consistent with genome-wide position location analysis, which established that di-methylated K4 is present in promoter and coding regions of genes in yeast but with a bias towards the coding region¹⁵. The ChIP analysis of specific genes shown here does not allow us to determine the relative amounts of methylation in promoters versus coding regions. However, the fact that tri-methyl is present in the coding region (this report), along with the enrichment of di-methylated K4 in the coding region¹⁵, suggests a role for K4 methylation in events associated with transcription elongation.

The findings presented here indicate that Set1 facilitates transcriptional activation and uncover a new level of regulation of K4 H3 function, by virtue of methylation status. Given that there are

many methylated lysines on histones H3 and H4, these observations suggest that the combinatorial potential for covalent modification on histones is far larger than previously suspected. It is no longer possible to surmise the transcriptional state of a gene based on whether a given lysine within its histones is methylated or not. The precise methylation state of the lysine is the true indicator of activity. □

Methods

SET protein purification and methyltransferase assay

SET proteins were tagged, expressed and purified as described previously¹⁶ from the C13 ABYS-86 strain (*Mata ura3 leu2-3 his3-112 pral-1 prb1-1 prc1-1 cps1-3*; D. H. Wolf). SET proteins (1 µg) were incubated in 50 mM Tris-HCl, pH 8.5, 20 mM KCl, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.05 mM dithiothreitol, 250 mM sucrose, 0.2% dodecyl-β-D-

maltoside buffer, with 2 μ l of 14 C-labelled SAM (NEC-363 adenosyl-L-methionine, S-(methyl- 14 C)) and 40 μ g of a mixture of soluble histones from calf thymus (H2A, H2B, H3 and H4; Sigma), 10 μ g of recombinant *Xenopus* wild-type H3, or 10 μ g of H3 deleted from the 26 first amino acids (Δ tail H3) for 1 h at 30 °C.

Expression profiling

The *set1 Δ* mutant and the isogenic UCC1001 strain were grown together in YPD medium at 30 °C to an optical density of 1.0 at 600 nm. Poly(A) RNA was isolated and reverse-transcribed incorporating amino-allyl dUTP. The resulting DNA probe was labelled with reactive Cy5 (mutant) or Cy3 (parent strain) dye and hybridized to a spotted complementary DNA microarray containing the yeast open reading frames as described previously^{17,18}. Microarrays were analysed using a GenePix4000A scanner and GENEPIX 3.0 software. Candidate genes regulated by Set1 were identified from duplicate experiments using a gene-specific error model described previously¹⁹. Genes reported to be activated by Set1 were downregulated in both profiles of the mutant *set1 Δ* strain. Microarray data were verified by northern blot analysis. Using a 1.5-fold cut-off, there are 480 downregulated and 885 upregulated genes.

Northern blots and ChIPs

Northern blots and ChIPs were done exactly as described²⁰. We used 5 μ l of tri-methylated K4 H3 antibody and 2 μ l of di-methylated K4 H3 antibody. For the ChIP experiments, the cells were grown in minimal medium lacking inositol (–ino), diluted to an optical density of 0.15 at 600 nm in minimal medium lacking inositol or the same medium but supplemented with 100 mg l^{–1} inositol (medium +ino), and grown to an optical density of 1.2 at 600 nm and then processed for chromatin preparation. Samples from medium without and with methionine were prepared in the same way except that the medium was supplemented with 30 mg l^{–1} of methionine where appropriate. The constitutive genes were scored from medium with inositol (*PPH3*) and medium with methionine (*HAM1* and *NUP170*). Primers for the PCR analysis are described in Supplementary Fig. 4.

Antibodies

The di-methylated K4 H3 and the tri-methylated K4 H3 specific antibodies are available from Abcam (<http://www.abcam.com>).

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Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair

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Although the acetylation of histones has a well-documented regulatory role in transcription^{1–4}, its role in other chromosomal functions remains largely unexplored. Here we show that distinct patterns of histone H4 acetylation are essential in two separate pathways of double-strand break repair. A budding yeast strain with mutations in wild-type H4 acetylation sites shows defects in nonhomologous end joining repair and in a newly described pathway of replication-coupled repair. Both pathways require the *ESA1* histone acetyl transferase (HAT), which is responsible for acetylating all H4 tail lysines, including ectopic lysines that restore repair capacity to a mutant H4 tail. Arp4, a protein that binds histone H4 tails and is part of the Esa1-containing NuA4 HAT complex, is recruited specifically to DNA double-strand breaks that are generated *in vivo*. The purified Esa1–Arp4 HAT complex acetylates linear nucleosomal arrays with far greater efficiency than circular arrays *in vitro*, indicating that it preferentially acetylates nucleosomes near a break site. Together, our data show that histone tail acetylation is required directly for DNA repair and suggest that a related human HAT complex may function similarly.

Four lysines, at positions 5, 8, 12 and 16, in the amino-terminal tail of histone H4 are reversibly acetylated *in vivo* in all eukaryotes⁵. The histone code hypothesis⁶ postulates that covalent modifications of histones such as acetylation, phosphorylation and methylation facilitate the binding of specific proteins to chromatin to alter transcription and possibly other events in DNA metabolism. HATs associate physically with the Ku70 DNA repair protein⁷ and with the ORC replication initiation complex^{8,9}, which suggests that histone acetylation may indeed be important in processes other than transcription.

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Mutations of yeast histone H4 in which all four tail lysines are replaced by glutamines (mutant *hhf1-10* (4K → Q)) cause a pronounced defect in genome integrity and engage the RAD9 checkpoint^{10,11}. To determine whether the genomic instability observed in *hhf1-10* might result from endogenous damage or a failure of DNA repair, we tested the *hhf1-10* mutant for sensitivity to several DNA-damaging agents, including camptothecin (CPT) and methyl methane sulphonate (MMS), which induce DNA double-strand breaks (DSBs), and ultraviolet radiation, which causes the formation of intrastrand photoproducts. The *hhf1-10* mutant was found to be markedly hypersensitive to both CPT and MMS (Fig. 1a), indicating the presence of a defect in DNA DSB repair. In contrast, the *hhf1-10* mutant was not even modestly hypersensitive to ultraviolet radiation (Fig. 1b), which causes DNA damage that is repaired through a separate pathway. These results show that the *hhf1-10* mutant is not globally defective in all DNA repair processes arising from an altered chromatin structure.

If lysine acetylation *per se* is required to repair damage caused by DSB-inducing agents, we reasoned that adding a single lysine to the *hhf1-10* tail, perhaps even in an ectopic location, might restore wild-type resistance to these agents, because a single lysine (but not a single arginine) is sufficient to reverse the RAD9 checkpoint arrest¹¹. Six mutants containing a single lysine were tested for CPT sensitivity: four mutants had a lysine in one of the natural positions in the H4 tail and two had an ectopic tail lysine. Adding a lysine into

five of the six positions in the H4 tail completely rescued the CPT hypersensitivity observed in the *hhf1-10* mutant (Fig. 1c), whereas lysine addition at position 16 only partially rescued hypersensitivity to DSB agents. Thus, a principal pathway for repair of DSB damage seems to require the presence of a single H4 tail lysine.

Critical to this interpretation is the prediction that the ectopic lysines in *hhf1-25* and *hhf1-35* will be capable of *in vivo* acetylation. To test this prediction, cell extracts from wild-type, *hhf1-10* (4K → Q), *hhf1-25* (4K → Q, + 1K) and *hhf1-35* (4K → Q, + 1K) strains were immunoblotted with an antibody against acetylated H4. Extracts from both *hhf1-25* and *hhf1-35*, but not the *hhf1-10* (4K → Q) control, showed a clear signal at the H4 position (Fig. 1d), indicating that the ectopic lysines are acetylated *in vivo*. This is consistent with the idea that lysine acetylation is required for the correct repair of DNA DSBs.

We reasoned that if *hhf1-10* is defective for DSB repair because lysine acetylation is blocked by mutation, then mutation of the HAT that acetylates the H4 lysines should also result in defective DSB repair. We tested mutants in several HATs that are not essential for DNA damage sensitivity including *HAT1*, *GCN5* and *SAS2*; none of these was hypersensitive to CPT (data not shown). We then examined the essential *ESA1* next because this HAT prefers H4 as a substrate *in vitro* and because loss of *ESA1* function has been reported to trigger the RAD9-dependent DNA damage checkpoint¹². We generated mutations in the chromosomal *ESA1* gene and scored colonies for both temperature-sensitive growth and CPT and MMS sensitivity at the permissive temperature. A subset of *esa1* mutants was specifically hypersensitive to CPT and MMS, whereas other temperature-sensitive mutants were not. Cells carrying the *esa1-L357H* allele did not grow at 37 °C but had wild-type resistance to CPT and MMS at 30 °C. By contrast, cells expressing the *esa1-1851* allele did not grow at 37 °C and were markedly hypersensitive to CPT and MMS at 30 °C (Fig. 2a). Thus, at the permissive temperature these two alleles separate the DSB repair function of *ESA1* from its essential function.

To determine whether the DSB repair defect in *esa1-1851* is reflected in the level of H4 acetylation, we examined the amounts of acetylated protein in both mutants by western blotting. The *esa1-1851* CPT hypersensitive mutant shows a loss of H4 acetylation even at the permissive temperature, whereas the *esa1-L357H* CPT resist-

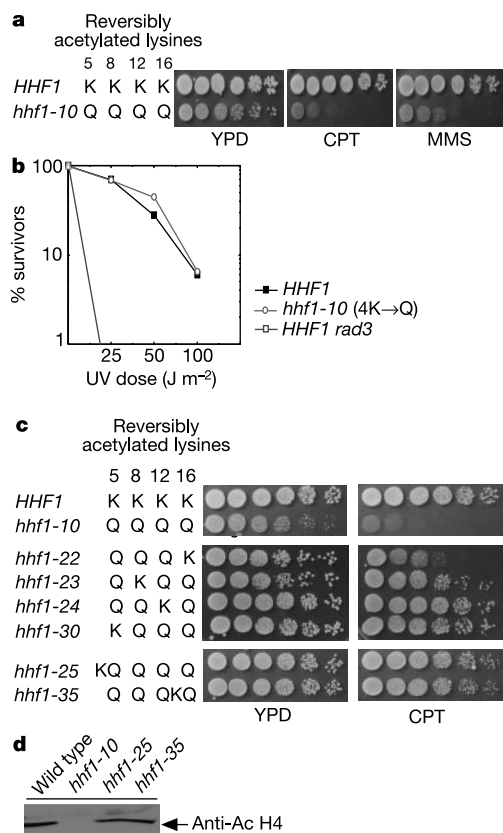


Figure 1 *hhf1-10* is hypersensitive to DSBs and addition of a single lysine to *hhf1-10* rescues CPT hypersensitivity. **a**, *HHF1* and *hhf1-10* strains were serially diluted on YPD, CPT (40 μg ml⁻¹) and MMS (0.030%) plates. **b**, *HHF1*, *rad3*, *hhf1-10* strains were irradiated using a Stratalinker ultraviolet (UV) source for various times and plated on YPD, incubated for 3 d and counted for surviving colonies. **c**, Strains were diluted on YPD and CPT (40 μg ml⁻¹) plates. **d**, The lysine inserted in *hhf1-25* and *hhf1-35* is acetylated *in vivo*. Cell extracts were resolved by SDS-PAGE and immunoblotted with antibody against acetylated H4 peptide.

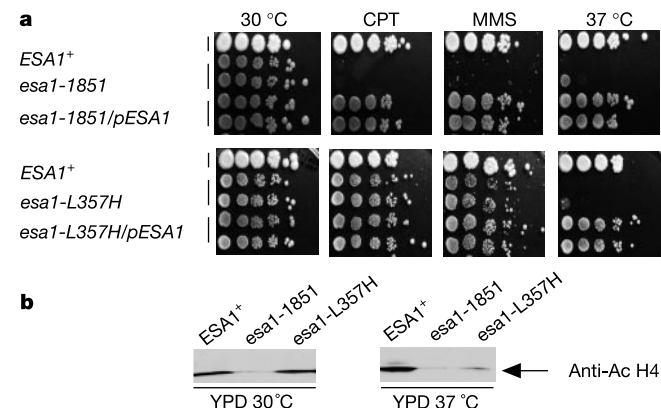


Figure 2 *ESA1* is required for H4 tail acetylation and for DSB repair. **a**, Histone H4 is hypoacetylated in *esa1* mutants. Cell extracts of *esa1* mutants grown at the permissive and non-permissive temperature were blotted with antibody against hyperacetylated histone H4. **b**, Conditional *esa1* alleles. The conditional *esa1-1851* allele gives rise to hypoacetylation of H4 at the permissive temperature and causes hypersensitivity to DSB-inducing agents. The *esa1-L357H* allele is equally sensitive to temperature, but shows no hypersensitivity to DSB agents or H4 hypoacetylation at the permissive temperature. *esa1-1851/pESA1* and *esa1-L357H/pESA1* are *esa1* mutants with a wild type plasmid re-introduced.

ant allele does not (Fig. 2b, left). At the nonpermissive temperature, H4 acetylation is diminished in both mutants, in agreement with reports that *Esa1* is responsible for most nucleosomal H4 acetylation *in vivo*¹³. Thus, the acetylation status of histone H4 at 30 °C correlates with cellular DSB repair capacity. These data also showed that *ESA1* is required both to acetylate the lysines in the H4 tail *in vivo* and for DSB repair. Similar to the *hhf1-10* mutant, the defect in the *esa1-1851* strain is very specific because we observed no marked hypersensitivity to ultraviolet damage (data not shown). Notably, the human homologue of *ESA1*, TIP60, also seems to be involved in DSB repair through an unknown mechanism¹⁴.

There are at least two pathways by which cells can repair DSBs: homologous recombination and non-homologous end joining (NHEJ). Much evidence indicated that homologous recombination was not defective in the *hhf1-10* mutant (Supplementary Information Table 1). To examine NHEJ, we used a standard plasmid recircularization assay^{15–17}. In this assay, the DNA around the DSB is located in the *kan^r* gene and is therefore not homologous with sequences on any of the yeast chromosomes. Thus, the DSB is repaired solely through NHEJ. We found that the *hhf1-10* mutant was highly defective in NHEJ, achieving only 20% of the repair of the wild-type strain (Supplementary Information Fig. 1).

Unexpectedly, mutants containing a single lysine at position 5, 8, 12 or 16 all showed a defect in NHEJ similar to that of the *hhf1-10* mutant (data not shown), which indicates that acetylation of more than one lysine is required for NHEJ. Consistent with this, adding a single ectopic lysine to the *hhf1-10* mutant tail did not suppress the defect in NHEJ (data not shown), despite the fact that a single lysine could restore the main DSB repair pathway (Fig. 1c). This showed that there are two distinct repair pathways that are defective in *hhf1-10*: an NHEJ pathway that requires acetylation of more than one of the H4 tail lysines but has a minor role in the cellular resistance to CPT or MMS; and a distinct pathway that requires acetylation of any one of the H4 tail lysines and is the principal determinant of DSB repair capacity. Only this second pathway was restored by adding a single ectopic lysine. Because this pathway was important in the repair of CPT-induced damage, which results in DSBs specifically at

the replication fork¹⁸, and not that caused by ionizing radiation, which results in random DSBs, we called this pathway the 'replication-coupled pathway'.

The *ESA1* gene is the primary HAT required for acetylation of the lysines in H4 (Fig. 2b); therefore, it might be required for one or both DSB repair pathways. To address this issue, we carried out NHEJ assays on the *esa1* temperature-sensitive mutants grown at the permissive temperature. Both mutants were defective for NHEJ, consistent with the requirement of *ESA1* for H4 acetylation (Supplementary Information Fig. 1). One of the *esa1* temperature-sensitive mutants was markedly hypersensitive to CPT, which strongly suggests that it also has a defect in the replication-coupled pathway.

To understand further the replication-coupled pathway, we carried out a genetic screen to identify genes whose overexpression restored camptothecin resistance to *hhf1-10*. A class of suppressors, represented by seven independent clones, carried the gene for the histone tail-binding protein Arp4 (Supplementary Information Fig. 1). This protein binds the wild-type histone H4 tail sequence *in vitro*¹⁹, but not an H4 tail in which the four conserved lysines are changed to glutamines (that is, *hhf1-10*)²⁰. We propose that overexpression of Arp4 drives its association with the mutant *hhf1-10* tail, partially restoring the DSB repair pathway. Notably, Arp4 and *Esa1* are both members of the NuA4 HAT complex²⁰ and thus independent lines of investigation have implicated the NuA4 HAT complex in DNA repair. The replication-coupled DSB repair pathway involves at least two components of NuA4, the direct histone tail-binding protein Arp4, and the only catalytic subunit of the HAT complex, *Esa1*. These results suggest a model in which the Arp4-containing complex binds to chromatin at a replication-coupled DSB to facilitate DNA repair.

To test this model directly, we examined the recruitment of Arp4 to the site of a specific DSB by *in vivo* crosslinking and chromatin immunoprecipitation (CHIP). A single DSB was created on chromosome III by inducing expression of the site-specific homothallic (HO) endonuclease in strains expressing a Myc-tagged Arp4 protein. We amplified DNA isolated from the anti-Myc antibody immunoprecipitates by polymerase chain reaction (PCR) using primers adjacent to the HO break site (0.6 kilobases), or primers located on a different chromosome as a control (Fig. 3a). Hdf2 (Ku80), a protein required for efficient NHEJ, is recruited to an HO-induced DSB by CHIP and serves as a positive control in these experiments²¹. As expected, there was a specific increase in amounts of Hdf2 near the DSB site 90 min after inducing the endonuclease

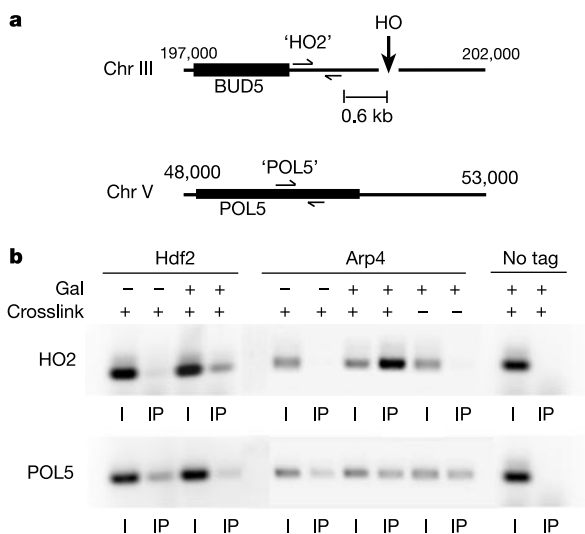


Figure 3 Arp4 is recruited to a DSB site *in vivo*. **a**, Location of the primers used in the chromatin immunoprecipitation (CHIP) assay relative to the HO cleavage site. **b**, CHIP experiments were carried out on strains containing Myc-tagged Arp4, Myc-tagged Hdf2, or no tag. Strains were grown in galactose for 45 or 90 min to induce expression of HO endonuclease. After induction, a substantial increase was observed in the localization of Arp4 and Hdf2 at DNA adjacent to the break site (HO2), but not to a distant site (POL5). I, input; IP, immunoprecipitate.

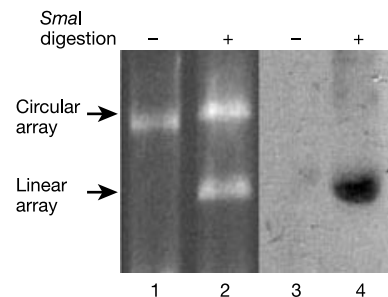


Figure 4 NuA4 preferentially acetylates linear nucleosomal arrays. Nucleosomes were reconstituted on covalently closed circular plasmid DNA and a portion was then linearized by digestion with *SmaI* endonuclease. Histone acetylation by NuA4 was assayed using either the circular nucleosomal template alone (*SmaI* -) or an equal mixture of circular and linear templates in a competition reaction (*SmaI* +). The reactions were separated by agarose gel electrophoresis and visualized with ethidium bromide for plasmid DNA (lanes 1 and 2) or by fluorography for histone acetylation (lanes 3 and 4). The locations of the circular and linear nucleosomal arrays are marked.

(Fig. 3b). There was also an increase in Arp4 crosslinking near the HO break site after endonuclease induction but no increase in crosslinking at the control site. A no-crosslinking control indicated that the Arp4 association with the break site occurs *in vivo* (Fig. 3b). These results show that within 90 min Arp4 is recruited specifically to a chromosomal locus in response to a DNA DSB and suggest that Arp4 and the associated HAT, Esa1, probably have a direct role in the repair process.

Because these results strongly implicated the NuA4 complex in the replication-coupled DSB repair pathway, we examined whether purified NuA4 complex might recognize a DSB in the context of chromatin *in vitro*. Nucleosomes were first reconstituted onto a covalently closed circular DNA template to form a nucleosomal array. A portion of this material was then linearized by digestion with *Sma*I, which created a DSB and a template with free chromatin ends. These circular and linear nucleosomal templates were then assayed in competition HAT reactions using purified NuA4 and [³H]acetyl-coenzyme A (CoA). These assays showed that purified NuA4 complex has a marked preference for nucleosomal histones in the linear array template and negligible activity on the circular array template (Fig. 4, lanes 2 and 4 contained equal amounts of circular and linear nucleosomal arrays present in the same reaction). A clear preference for the linear array could be seen in lane 4, as this form shows a much greater incorporation of [³H]acetate from [³H]acetyl-CoA than does the circular array. Thus, the NuA4 complex has an inherent ability to recognize a DSB in the context of chromatin. This is consistent with the observation that NuA4 can acetylate the interior portion of a linear array only if drawn there by an activator, but it can act on the ends without an activator²².

Our data indicate that the acetylation of histone H4 participates directly in one or more steps required to repair broken chromatin. First, Arp4, a histone tail-binding protein that is sensitive to mutations in H4 lysines, is specifically recruited to the site of a DSB *in vivo*. Second, NuA4, a HAT complex that acetylates histone H4 lysines, preferentially acetylates linear chromatin templates *in vitro*. Third, northern blot analysis of the expression of DNA damage repair genes, including *RAD50*, *MRE11*, *XRS2*, *DNL4*, *RNR1* and *RNR3*, shows that the amounts of their messenger RNAs are not altered in either *hhl1-10* or the *esa1* mutant strains (data not shown). Last, comparison of the transcription profiles of *hhl1-10* and *hhl1-25* using whole-genome oligonucleotide microarrays (Affymetrix) shows that none of the genes involved in any DNA DSB repair pathway has substantially decreased expression (Supplementary Information Table 2).

The exact nature of the repair defect in the replication-coupled pathway remains to be determined. An intriguing possibility is the poorly understood role of the recombination-mediated restart of stalled replication forks, which is well documented in bacterial cells²³ but poorly understood in eukaryotes. The histone code hypothesis⁶ predicts that modifications such as acetylation and phosphorylation are likely to function as signals for recruiting specific repair complexes, rather than as simple modifiers of the charge on chromatin. The involvement of Arp4, which binds only to acetylated H4 tails²⁰, in the repair of DSBs supports the histone code hypothesis⁶. The code may be complex because H2A phosphorylation also seems to be involved in NHEJ²⁴. Our data show that histone acetylation is required for DNA repair in addition to its well-established role in transcription, and suggest that histone acetyltransferases are likely to function in other aspects of DNA metabolism such as recombination and replication. □

Methods

Drug sensitivity

To determine sensitivity to CPT and MMS, yeast strains were grown overnight, diluted to an absorbance at 600 nm (*A*₆₀₀) of 0.2–0.3 and grown to *A*₆₀₀ ≈ 1.0 before being plated on yeast peptone dextrose (YPD) medium at tenfold dilutions. We incubated all plates for 2–3 d at 30 °C.

NHEJ assays

Yeast strains were grown overnight, diluted to *A*₆₀₀ ≈ 0.2–0.3 and grown to *A*₆₀₀ ≈ 0.6–0.7 before being transformed with digested or undigested plasmid DNA as described¹⁷. Plasmid DNA was digested with *Hind*III for 3 h and extracted with phenol before transformation.

Western blots

We grew 10-ml cultures of the histone mutant strains to *A*₆₀₀ ≈ 1.2–1.6 and lysed them in HSB buffer (45 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA and 0.5% Nonidet P-40) plus 20 mM sodium butyrate by bead lysis homogenization. Total protein concentration of each of the samples was determined by the Bio-Rad protein assay and the amount of protein was normalized and loaded onto a 15% agarose gel. We carried out western blotting using an antibody against hyperacetylated histone H4 (a gift from D. Allis).

Chromatin immunoprecipitation

We carried out CHIP assays as described²¹. Fifty-microlitre cultures of Arp4-Myc, Hdf2-Myc and untagged strains were grown overnight to log phase, and expression of HO endonuclease was induced for 90 min. Cultures were then crosslinked for 60 min and extracts prepared for immunoprecipitation with antibodies against Myc. DNA isolated from the immunoprecipitates was used in PCR reactions containing the primers shown in Fig. 3a.

Detection of NuA4 targeting by fluorography

Fractions of NuA4 eluted from a Superose 6 column were purified and assayed for their ability to acetylate nucleosomal substrates as described²⁵. We carried out nucleosome reconstitution and HAT assays by a protocol modified from ref. 22. The plasmid pG5E4T (ref. 26), was used for reconstitution and NuA4 acetyltransferase assays. Circular plasmid DNA in 2 M NaCl was mixed with a 1:1 molar ratio of core histones purified from HeLa cells and reconstituted *in vitro* by step dilution as described²⁷. The efficiency of reconstitution was determined by comparing the electrophoretic mobility of the nucleosomal array with that of naked DNA separated on 1.2% agarose, 1 × Tris acetate EDTA (TAE) gels for 240 V h⁻¹ and then stained with ethidium bromide. Reconstituted templates were linearized with *Sma*I for 1 h at 37 °C and then chilled on ice. To some reactions, we added an equal volume of uncut reconstituted template. Each reaction was incubated together with 1 μl of NuA4 complex and 0.25 μCi of [³H]acetyl-CoA (Amersham) and incubated for 15 min at 30 °C. The whole reaction was separated on a 1.2% agarose, 1 × TAE gel for 240 V h⁻¹. The gel was then fixed by agitation for 1 h in 10% acetic acid and 10% methanol, developed with EN3HANCE for 3 h (NEN Life Science Products), rinsed for 1 h in distilled deionized water, dried and exposed to film.

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Competing interests statement

The authors declare that they have no competing financial interests.

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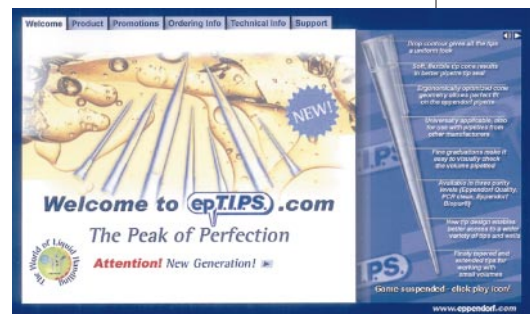
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When Laura Coruzzi finished her postdoc at New York's Mount Sinai School of Medicine in 1981, her scientific future looked uncertain. Grant money had dried up, owing to federal cutbacks. Even established faculty had problems finding funding. Such a grim outlook often leads people to look for alternatives, and Coruzzi was no exception.

Through a combination of personal contacts and serendipity, she met Leslie Misrock, a partner at Pennie and Edmonds, a New York law firm that was looking to capitalize on the anticipated boom in biotech patents. She joined the firm, along with Jennifer Gordon, a biochemist from the Massachusetts Institute of Technology, who didn't fancy a future standing next to a fermenter. The 'patent twins' clerked for the firm (which covered the cost of law school) by day, attended Fordham University School of Law by night, and studied whenever they could.

They are now both partners in the firm, and their experience is instructive for any scientist considering a career beyond the bench. First, there's location. Being in a city like New York, with a concentration of academic institutions (see Regions, page 4) and science-related business opportunities outside the ivory tower, increases the chance of a successful move.

Then there's hard work. Few people who have slogged through a PhD welcome the thought of more formal education. But to cross over successfully, a law degree, MBA or other professional qualification is often helpful. Finally, there's expectation. Misrock convinced Coruzzi and Gordon that there'd be a need for their services, so they took the plunge. And their hard work paid off. Scientists who follow a similar plan can be equally successful, even if they choose a career other than law and a city other than New York.

Paul Smaglik

Naturejobs editor



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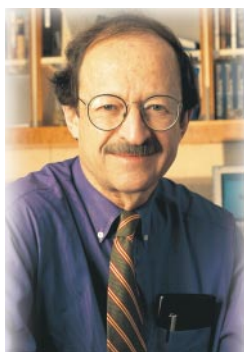
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SCIENTIFIC EVENTS



Building cooperation New York

Harold Varmus says that cooperation between New York's academic institutions is already reaping dividends.



Within the next five years, New York City's six major medical schools and four research universities will add between 140,000 and 190,000 square metres of lab space. That's enough to provide 1,000 new investigators with 100 square metres each, with plenty of room left over for core facilities such as instrumentation and computing. But even before those projects are complete, the universities and hospitals will have built something else, perhaps more valuable but less tangible — solid ties between institutions that once saw each other as competitors.

Both building booms have been engineered to attract world-class scientists, and help them work beyond institutional walls once they arrive. The core of cooperative activity in New York is Manhattan's Upper East Side, which holds the Memorial Sloan-Kettering Cancer Center (MSKCC), the Rockefeller University and the Weill Medical College of Cornell University. These three institutions have already had some success in collaborations on research, training and housing. But whether they can together build up biotech in the city (see "Biotech dreams", opposite) is another question.

Harold Varmus, president of MSKCC and an architect of the tri-institutional alliance, points out that both building efforts are already reaping dividends. The new building will be partly open in 2005 and completely open in 2007. It will

effectively double MSKCC's research space and drive recruitment, says the Nobel laureate and former director of the US National Institutes of Health.

It has already helped attract Thomas Kelly from Johns Hopkins University to lead basic science research at MSKCC's research arm and Robert Wittes from the US National Cancer Institute to lead clinical investigation at the associated hospital. They will help Varmus recruit another 40 to 50 principal investigators.

CORE COOPERATION

Varmus describes alliances within New York City research institutions as concentric circles, like the layers of skin on an onion. The first layer, from his point of view, is the fostering of bench-to-bedside research from the basic-science-oriented Memorial Sloan-Kettering Institute and the clinically oriented cancer centre. He expects Kelly and Wittes to lead that charge. And in July, computational-biology expert Chris Sander came aboard and will build a staff of 70. The next layer is the tri-institutional arrangement between MSKCC, Cornell and Rockefeller. This relationship combines building construction, recruitment and training. On the construction side, one project addresses one of the toughest demands of living in New York if you're a graduate student or postdoc — the shortage of affordable housing close to the lab. To meet that need, the three institutions have started building subsidized housing on Roosevelt Island, east of Manhattan. Several hundred units will open in the summer of 2003 and over 1,000 people may eventually be housed on the island.



Biotech dreams

The biotech industry would seem to be a natural fit with Manhattan's biomedical research institutions. But the area's biotech scene lacks two vital ingredients — space and a track record of success.

In Manhattan, there's only one incubator for biotech start-ups, Columbia's Audubon Business and Technology Center, with 9,300 square metres, and entrepreneurs have had to wait months for space.

Harold Varmus, president of the Memorial Sloan-Kettering Cancer Center (MSKCC) wants to identify 100,000 square metres in Manhattan for biotech. But there are no firm plans yet.

Gerald Fischbach: joint appointments can avoid competition.

The lack of a local biotech success story — the New York equivalent of a Genentech or a Millennium — doesn't help. The therapeutic antibody company ImClone might have been one, but recent troubles there (see *Nature* 417, 584–586; 2002), badly affected the reputation of both the company and biotech as a whole.

But there are glimmers of hope. Drug-discovery companies OSI Pharmaceuticals of Long Island and Regeneron of Westchester have prospective drugs working their way through the clinical pipeline. Another young company, Mojave Pharmaceutical of Hawthorne, New York, is working on immunotherapeutic techniques developed at MSKCC.

At least one company needs to get a product to market to create demand for expansion, says Karin Duncker, executive director of the New York Biotechnology Association. "What we need is some good news."

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research options are attracting top students. And combining forces makes sense scientifically as well. "It gives you much greater potential for attacking a scientific problem," Gotto says. "It's a tearing down of the walls of the ivory tower."

Like MSKCC, Cornell is counting on construction to attract both students and faculty. Last year they completed a US\$220 million programme to turn an old hospital building into a research facility and to attract 30 scientists to fill it. Most of the positions have been filled, but these professors are now hunting for graduate students and postdocs. The university's next major project is to build and staff a US\$750-million clinical research centre.



Weill's Antonio Gotto looks beyond the ivory tower.

OUTER LIMITS

Although the tri-institute agreements serve as the core, another cooperative effort may have even wider effects. Over the past few years, 39 research institutions in and around New York have banded together under the umbrella of the Academic Medical Development Company (Amdec) to undertake even larger research projects, to do training in entrepreneurial activities, and to exert bulk purchasing power for expensive items such as microarrays.

One of the group's largest projects to date is a cancer epidemiology study in which 20,000 people from a variety of ethnic backgrounds have been enrolled. They have donated blood samples, which are being stored at a biorepository at Columbia's College of Physicians and Surgeons, and their health will be monitored over the next 10 years.

Relationships between institutions also come about more informally. Albert Einstein College of Medicine in the Bronx is focusing on the development of biomedical technology. It is building an innovations centre that will bring together physicists, biologists, engineers and computational specialists to develop new imaging and diagnostic equipment. Even though the facility will not be complete until 2005, Einstein scientists are already cooperating with other New York institutions. They are collaborating with MSKCC in the use of microarrays to analyse gene expression in cancer, and with Columbia University, whose campus is located in Manhattan's Upper West Side, in the use of algorithms to look for correlations between data that exist in different dimensions, says Robert Singer, professor of cell biology at Einstein.

But even with the best intentions, institutions can still bump heads. Gerald Fischbach, dean of Columbia's medical school, applauds progress on their Irving Cancer Research Center, which will open in spring 2003. But he hopes it won't overlap with MSKCC's own expansion. "I hope we're not competing," Fischbach says, joking that conflict with Harold Varmus, his old boss at NIH, could prove contentious. "We're both tough guys," he says. But joint appointments between the rivals could iron out the difference. "There's not infinite expertise in this world," says Fischbach. Not even in New York.

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On recruitment, the three institutes will fund and fill 12 tri-institutional professorships, with the first going to Stan Leibler, a biophysicist at Rockefeller. The arrangement will allow those professors to work with graduate students in any or all of the institutions. Future appointments are anticipated in computational biology and developmental biology, and will probably come from outside the New York area.

In terms of training, the three have combined forces in two areas — the established MD/PhD programme, and a new training programme in chemical biology. The MD/PhD programme provides the MD training at Cornell, but allows physicians and scientists in training to do research at any of the three institutions. The chemical biology programme, which started this autumn, trains graduate students at Cornell's Ithaca campus, but then allows them to do their thesis research at any of the three Manhattan institutions. Antonio Gotto, dean of Weill Medical College, says that the wider